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13. ABSTRACT ***(Maximum 200 Words)***

To assess, maintain, or improve a soldier's physical/physiological/psychological capability to function effectively under environmental and operational stress and to minimize adverse effects of stress on health safety and performance, the PBRC performs the following eight research tasks: 1) Clinical Laboratory for Human and Food Samples performs laboratory analysis of samples from studies conducted by the U.S. Army Research Institute of Environmental Medicine (USARIEM) and at PBRC in Tasks 4 and 8. 2) Stable Isotope Laboratory performs analyses to measure the energy expenditure and body composition of soldiers during prolonged field exercise and at PBRC in Tasks 4 and 8. 3) Stress, Nutrition and Mental Performance Laboratory continues multidisciplinary basic research studies of the interactions of stressors and nutrition on mental performance parameters in an animal model. This lab collaborates with Task 7 which evaluates immune function in the stressed animal model. 4) Stress, Nutrition and Work Performance uses human subjects to develop nutritional strategies to improve physical performance under intense physical stress. 5) Nutrient Database Integration Laboratory supports the Military Nutrition Division and PBRC research studies by providing dietary intake and analysis support. 6) Enhancing Military Diets targets health promotion through improved nutrition in basic combat training. 7) Stress Nutrition and Immune Function Laboratory provides special tests of immune function in collaboration with Task 3 to evaluate immunologic change in a rat model. 8) Metabolic Unit Project allows new inpatient protocols to address specific issues in nutrition and metabolism that are important to the military mission.

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

David J Ryan 7/28/00

PI - Signature Date

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ANNUAL REPORT
US ARMY GRANT #DAMD 17-97-2-7013
July 1, 1999 - June 30, 2000

Introduction

On April 1, 1997, Grant #DAMD 17-97-2-7013, **Military Nutrition Research: Eight Tasks to Address Medical Factors Limiting Soldier Effectiveness**, was awarded to the Pennington Biomedical Research Center (PBRC) to address the following hypothesis: Soldier effectiveness in conditions of environmental, physiological and psychological stress can be enhanced by nutritional measures. The overarching rationale of the project is that better understanding of the nutritionally related mechanisms induced by stress will allow countermeasures to be designed and tested.

The goal of this research is to assess, maintain, or improve a soldier's physical/ physiological/psychological capability to function effectively under environmental and operational stress and to minimize adverse effects of stress on health, safety and performance.

Technical Objective

This research continues the research relationship between PBRC and USARIEM over a five-year period. Those research relationships were established under prior cooperative agreements, #DAMD 17-88-Z-8023, "The effect of food, diet and nutrition on military readiness and preparedness of military personnel and dependents in a peace time environment," and #DAMD 17-92-V-2009, "Military nutrition research: six tasks to address medical factors limiting soldier effectiveness."

The project allows for the continuation of the Clinical Laboratory for Human and Food Samples, Stable Isotope Laboratory, Menu Modification Project, and Nutritional Neuroscience Laboratory, all of which were initiated under Grant #DAMD 17-88-Z-8023. The project also expands the scope of research to allow for studies in humans of stress, nutrition and work performance, and for studies in humans and animals to evaluate the relationship of stress, nutrition and immune function. The grant provides a nutrient database laboratory. The project also allows for the utilization of PBRC's inpatient metabolic unit for a study designed by USARIEM investigators as detailed in the Metabolic Unit Project section.

Military Significance and Relevance to USARIEM Needs

The Stable Isotope and Clinical Laboratory methodologies are critical components of in-house military nutrition research of the U.S. Army Research Institute of Environmental Medicine. These extramural projects provide critical capabilities that do not exist in house, but are needed to fulfill the Army Surgeon General's responsibility to provide nutritional research support to the DOD and Nutrition RDT&E Program.

The Nutritional Neuroscience Laboratory expands our knowledge of the effects of stress and the mechanisms of stress-related performance decrements and explores the ameliorative effects and mechanisms of action of dietary-induced alterations in behavior and cognitive function. Advances in this knowledge are the basis for developing safe and effective nutritional strategies to sustain and enhance soldier performance under conditions of environmental or operational stress. The project specifically provides insight into the roles of corticotrophin releasing factor (CRF) and locus coeruleus (LC) noradrenergic mechanisms in mediating anxiety in rats exposed to restraint stress.

The Menu Modification Project fulfills military needs to promote health, maintain readiness and sustain soldier performance. The Nutrient Database Integration Laboratory supports USARIEM projects assessing food intake in the field.

The Metabolic Unit Project fulfills military need for an inpatient site for performance of specialized research utilizing the body composition assessment, energy expenditure assessment, metabolic kitchen services, and clinical laboratory expertise of PBRC.

This annual report describes progress during the third year of the grant. Discussions of the eight individual tasks funded under this grant follow.

I. Clinical Laboratory for Human and Food Samples

A. Introduction

The Clinical Research Laboratory and Food Analysis Laboratory continued to offer a broad spectrum of analytical tests in support of military nutrition research. The laboratory hired two new full-time accessioners and one part-time phlebotomist and continued to operate with the same testing and managerial personnel in place. The lab continued to receive specimens from army nutritional research. Some new tests were evaluated and put on-line and analyses were performed and completed for army studies. Results were compiled to returned to the army in electronic and hard copy form. The laboratory maintains certification by the College of American Pathologists (CAP) and the Health Care Financing Authority (HCFA). The lab is preparing for a CAP inspection in October 2000. The lab maintains certification by the Center for Disease Controls for lipid analyses. The Food Analysis Laboratory continued to analyze samples and work on test development.

The clinical laboratory performed testing for the following studies: SFAS-6, Ranger 4, Post Exercise Nutrient Supplementation study, Eccentric Exercise Study, Effects of Repeated Dosings of Caffeine on Vigilance, Combat Army Surgical Hospital Study, the Sergeant Major's Academy study, the Mangoday study, the in-house Bike Challenge Amino Acid study (Jeff Zachwieja), and the Navy study (Assessment of Energy Expenditure and Nutritional Status of Female Navy Personnel Onboard Ship).

B. Body

A new accessioner, Donald Lewis, was hired to replace the departed Jeff Copley. Donald began on January 18, 2000. A new part-time phlebotomist, Patricia Beasley and a new full time accessioner, Carla Milo, began work in April. Both have been trained and are now working on a routine basis. Patricia works in the morning and Carla works from 9-5:30. Mendy Richard was appointed as Business and Continuing Education supervisor to aid Dr. Tulley in business matters, including budgeting, workload, etc. Stacey Roussel was trained on the HPLC to perform amino acid analyses. Three new students were hired: Ashley Gray, Sourav Sengupta, and Stephanie Hall. Kyla Turpin and Leah Moore both graduated in June and resigned their positions as student workers.

Ranger 4 tests were completed for chemistry panel, BHBA, NEFA, lactate, thyroids, TSH, free T3, free T4, cortisol, testosterone, folate, and growth hormone. Pending are the vitamin A, vitamin E, and carotenoids.

SFAS-6 chemistries and the immunoassays (thyroids, TSH, free T3, free T4, cortisol, testosterone, folate, and growth hormone) were completed. Vitamin E, vitamin A, and carotenoids are still pending .

The laboratory completed testing for the Navy study under the direction of Dr. Hal Goforth called Post-Exercise Nutrient Supplementation Study (PENS). These included CK, cortisol, insulin, glucose, urine nitrogens, and IGF-1. Tests were run for alanine, glutamic acid, glutamine, and leucine by HPLC but some samples are still pending for amino acid analysis.

Analyses of chemistry panel including CK, IL-1, IL-6, TNF-α, CRP, and myoglobin were completed for the Eccentric Exercise Study (EES). Other tests to be performed include total antioxidant capacity, vitamin C, myosin heavy chain fragments (MHCF), and vitamin E. A method for myosin heavy chain fragments has yet to be found.

Multiple shipments were received and analyses for salivary melatonin and caffeine were completed for Harris Lieberman's study (Effects of Repeated Dosings of Caffeine on Vigilence).

The analysis of homocysteine for the El Paso and CASH Studies were completed and results sent to Natick.

Salivary melatonin analyses were completed for the Mangoday study and results returned to Natick.

Multiple shipments of samples from Harris Lieberman's study the Effects of Repeated Dosings of Caffeine on Vigilence (CSD) study were received and salivary caffeine and melatonin results were returned. One group of samples was missing from this study. We checked all our

ultralow freezers and were unable to locate it and believe that it was never sent to us. Army personnel cannot locate the missing samples.

Samples were received from the Navy study (Assessment of Energy Expenditure and Nutritional Status of Female Navy Personnel Onboard Ship). Tests performed and completed included homocysteine, erythrocyte glutathione reductase, erythrocyte transketolase, erythrocyte aspartate aminotransferase, apolipoprotein A1, apolipoprotein B, transferrin, 1,25 dihydroxy vitamin D, osteocalcin, total iron binding capacity, chemistry and lipid panel, vitamin B12, serum folate. Some samples leaked in transit, but most were salvaged. In addition, vitamin C samples were not processed correctly so that assay could not be performed. Vitamin A, vitamin E, and carotenoids are still pending awaiting method development. The only other test not completed is RBC folate. Samples were collected according to the protocol of Abbott for the IMx (a 1:21 dilution of sample with 1% ascorbic acid). Unfortunately, the Abbott test was removed from the market. We tried setting up RBC folate on our Immulite analyzer using the serum folate reagent. Results were not good using this instrument. Our plans are to return to our old method (Bio Rad RIA) for RBC folate; however, the method calls for a 1:21 dilution of sample with 0.4% ascorbic acid. Bio Rad does not know if the method will work with 1% ascorbic acid. Since the samples were already collected with 1% ascorbic acid we will investigate the accuracy of results using the Bio Rad method. The kit has been ordered and we will do this investigation ourselves. Data has been downloaded from the PBRC computer database and will be sent to Mark Kellogg.

A box of samples was received from the Arctic Norwegian Soldier study. We were instructed to wait to analyze these until all follow-up samples are received. A PBRC nutrition panel will be run on these when we are given permission to begin.

Troubleshooting on the amino acid method by HPLC was performed and it appears that the method is nearly ready for routine analyses again. The final wash was changed from acetonitrile to methanol and the run is again less than 20 minutes per sample. Pending amino acid analysis are samples from the Navy study of Hal Goforth (Post Exercise Nutrient Supplementation (PENS)). After that, additional amino acids will be validated and run for the Jeff Zachwieja Army study, BCAA. Control ranges need to be established and more amino acids need to be calibrated and verified.

It was decided that more help to perform method develop and sample analysis for our HPLC methods for Vitamins E and A, carotenoids, and amino acids is needed; therefore a position for an HPLC specialist was created and advertised. Two candidates were interviewed for the position. Upon hiring, this person should be able to complete method evaluation and perform analyses on our backlog of samples.

The HPLC method for carotenoids is continuing but not yet ready for use yet. Mendy Richard is working on this method development. As soon as linearities, precision, and recoveries are performed, we will analyze samples from Savannah, CASH, Ranger 4, and SFAS-6 studies, and the new Navy study (Assessment of Energy Expenditure and Nutritional Status of Female

Navy Personnel Onboard Ship-carotenoids; Navy and BCAA-amino acids). Vitamin A, E, and carotenoids are still pending for Savannah, CASH, Ranger 4, and SFAS-6.

We are developing a method of direct access to their data by the Army collaborators. Possible techniques to deliver this capacity include a terminal with Meditech software that would allow Army investigators to view completed results on samples for their studies. A problem with this is that it would be on a sample by sample basis and no compiled results would be obtainable. The other alternative is to provide access to a special database set-up by PBRC Computer Services upon completion of the results for a study. This would allow for direct access to compiled data; however, methods of ensuring the safety of the PRBR database would have to be a priority. Advantages of this would be that Army collaborators could order tests and print bar code labels for samples directly from the system. Access to results would also be available. Approval for access to the 1-800 PBRC telephone line has been obtained. This investigation will continue until a satisfactory conclusion is reached.

Dr. Tulley attended a meeting with the Army in Natick on June 8-9. The meeting was informative of upcoming new studies. These include the Marine Women Study, GH/IGF-1 Study, and Mark Kellogg's Filter Paper Investigation. It is anticipated that we will send specimen processors for the new Marine women's study to be done at Parris Island, South Carolina. New tests for fructosamine, CRF, Neuropeptide Y, Substance P will be set up for that study. Commercially available tests will be evaluated and methodology determined for these tests.

New tests investigated, validated, and put on line included: Interleukin 1 Beta, Interleukin 6, Tumor Necrosis Factor, myoglobin, C Reactive Protein (all on the DPC Immulite), red cell selenium (atomic absorption), glutathione peroxidase, red cell glutathione peroxidase (both on the Beckman CX5), IGF-1 (ALPCO ELISA), IGF-2 (DPC RIA), and homocysteine on the Abbott IMx.

A method for the analysis of cis and trans fatty acids was developed and put on-line in the Food Analysis Laboratory. Work was continued on a method for the analysis of dietary fiber.

C. Key Research Accomplishments

- Performed testing for studies in collaboration with military investigators and PBRC grant personnel, including:
  - SFAS-6
  - Ranger 4
  - Post Exercise Nutrient Supplementation
  - Eccentric Exercise Study
  - Effects of Repeated Dosings of Caffeine on Vigilance
  - Combat Army Surgical Hospital Study
  - Sergeant Major's Academy study

- Mangoday study
- The in-house Bike Challenge Amino Acid study (Jeff Zachwieja)
- Navy study (Assessment of Energy Expenditure and Nutritional Status of Female Navy Personnel Onboard Ship).

- Received samples for the Norwegian Arctic Army Study
- Validated methods of analysis and put testing on-line for:
  - Interleukin 1 Beta on the DPC Immulite
  - Interleukin 6 on the DPC Immulite
  - Tumor Necrosis Factor on the DPC Immulite
  - Myoglobin on the DPC Immulite
  - C Reactive Protein on the DPC Immulite
  - Red cell selenium on the Perkin Elmer graphite furnace atomic absorption instrument
  - Glutathione peroxidase on the Beckman CX5
  - Red cell glutathione peroxidase on the Beckman CX5
  - IGF-1 (ALPCO ELISA)
  - IGF-2 (DPC RIA)
  - Homocysteine on the Abbott IMx.
  - Trans and cis Fatty acids in food

D. Reportable Outcomes

Publications: see list of references in References section.

Presentations:

1. Richard Tulley, "The ABCs of Vitamin C: Analysis and Biochemical Correlations" – jointly sponsored meeting of the Texas section and Pediatric and Nutrition Divisions, American Association for Clinical Chemistry, Austin, TX, April 1999.
2. Richard Tulley, "The Relationship of Homocysteine & B Vitamins to Cardiovascular Disease. Roundtable. American Association for Clinical Chemistry, New Orleans, July 28, 1999.
3. Richard Tulley, "The ABCs of Vitamin C: Analysis and Biochemical Correlations". Roundtable. American Association for Clinical Chemistry, New Orleans, July 29, 1999.
4. Richard Tulley, "The Relationship of Homocysteine and B Vitamins to Cardiovascular Disease". Scientific Session. American Society of Clinical laboratory Scientists, New Orleans, July 30, 1999.
5. Richard Tulley, "The Relationship of Homocysteine and B Vitamins to Cardiovascular Disease". Mardi Gras Mambo Continuing Education Seminar, Pennington Biomedical Research Center, February, 2000.
6. Richard Tulley, "Relationship of Homocysteine and B Vitamins to Cardiovascular Disease". Joint Meeting of the Louisiana and Mississippi Societies for Clinical Laboratory Scientists, Jackson, Mississippi, April 2000.

7. Jennifer Rood, "Carotenoids and Their Role in Cancer Prevention and Cancer, "American Association for Clinical Chemistry", Roundtable, July, 1999.

E. Conclusions

The clinical and food analysis laboratories performed valuable service to the army by performing testing for military nutrition research. New methods of analyses were developed and put on line in both labs.

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II. Stable Isotope Laboratory

A. Introduction

The research conducted by the Stable Isotope Laboratory is in the area of energy and water requirements, and changes in body water, of soldiers, often under harsh environmental conditions. The method used to determine energy requirements is the doubly labeled water (DLW) technique, which involves oral administration of water labeled with the stable isotopes, ^{2}H and ^{18}O. Saliva and urine samples are then obtained for periods of 4-14 days, longer with redosing. Water intake can be determined using only the ^{2}H labeled water. The use of doubly labeled water for measurement of energy expenditure was developed as a field technique for use in small animals (1). The method is based on the premise that after a loading dose of $^{2}H_{2}{}^{18}O$, ^{18}O is eliminated as CO_2 and water, while deuterium is eliminated from the body as water. The rate of CO_2 production, and, hence, energy expenditure, is calculated from the difference of the two elimination rates. The only requirement of subjects is to give urine and saliva specimens before and after drinking an initial dose of $^{2}H_{2}{}^{18}O$, and then return in one to two weeks to give a final urine specimen. During the period between the two urine and saliva samplings, subjects are free to carry out their normal activities and are not required to maintain extensive diaries. The doubly labeled water method has been extensively validated in humans under controlled settings (2), but there are confounding factors that need to be considered in field studies, particularly in Army Field Studies. Among these are change in location or food and water supply immediately preceding, or during an energy expenditure study. These changes may cause a change in baseline isotope abundance and, therefore, interfere with the accuracy of the energy expenditure measurement. This has occurred in a previous field training exercise involving the study of the MRE and RLW rations (3). This is a particular problem with studies such as the Ranger Training Studies (4), in which soldiers are moved to different parts of the country during the study. Therefore, a group not receiving labeled water must be followed to make any corrections in baseline isotope shifts.

Hydration status is another main focus for some Army studies. Using the cheaper and more readily available deuterium tracer, either changes in total body water (5,6) can be followed during a study, or water turnover (intake) (7,8) can be measured during a study.

One advantage of the DLW method is that it uses stable isotopes so there is no radiation exposure. The method uses two heavy isotopes of water, which are naturally occurring in food and water. There are no known side effects of either isotope at the doses given in DLW studies and has been used extensively to study energy expenditure during pregnancy (10,11) lactating women (12), and infants for measurement of energy expenditure and human milk intake (13-15).

The Stable Isotope Lab was involved in several Army research projects during the current year. These are described below.

B. Body

Stable isotope studies were completed for 3 studies and a 4th study was begun. The first study employing doubly labeled water was an Infantry Officer Training Course, conducted at Quantico during March 1999. The isotope baseline shift in the undosed placebo group was given in a table in the 8th Quarterly Report. These baseline shifts were used to adjust the enrichments in the subjects receiving the doubly labeled water. To convert CO_2 production to energy expenditure we estimated that the RQ of these subjects was 0.80, as they were given only one MRE per day and would be burning considerable body stores of fat to attain energy balance, but they did get extra foods later in the study. The measured energy expenditures of these subjects was quite high. This was expected since they were carrying around 100-150 lbs. and were getting very little sleep.

Energy Expenditures

Subject #	EE, kcal/day	Body Wt, kg	Kcal/kg/day
Subject 2	5110	66.1	77.3
Subject 4	5691	70.3	81.0
Subject 5	6891	73.4	93.9
Subject 6	7757	93.9	82.6
Subject 8	6055	79.2	76.5
Subject 9	7151	82.9	86.3
Subject 11	5918	80.8	73.2
Subject 12	6889	80.6	85.5
Subject 13	6107	72.5	84.2
Subject 14	7147	89.5	79.9
Average→	**6472 ± 816**	**78.9 ± 8.6**	**82.0 ± 5.9**

Isotope analyses were completed for the doubly labeled water study for the Summer Quantico Study. There were no shifts in baseline isotope abundance in the 5 subjects who received tap water. Therefore, no adjustments were necessary in the ten subjects who received the DLW dose. The subjects received only one MRE and 5 packs of a carbohydrate beverage during the study. Therefore they only received just under 2300 kcal per day, and were in substantial negative energy balance. To calculate the energy equivalent of CO_2, the macronutrient content of the MRE, the carbohydrate beverage, plus available glycogen stores were calculated, and body stores of protein and fat (assumed 20% protein and 80% fat) were estimated. This led to an RQ of 0.804 (including protein), giving an energy equivalent of CO_2 of 5.769 kcal/L CO_2. The calculated total body water (TBW) and total daily energy expenditures (TDEE) are given in the following table.

Subject #	TBW (kg)	TDEE (kcal/d)
#1	52.8	3622
#2	40.9	3324
#3	50.9	4190
#4	44.3	3435
#5	58.7	4293
#6	54.2	5029
#7	49.3	5006
#9	50.4	4744
#12	45.5	3384
#13	48.2	4044
Avg	49.5	4107
SD	5.2	661

Isotope analyses and calculations for the 9-day simulation of a disabled submarine (DISSUB) with eight volunteer "survivors" conducted in the NATICK chamber facility are complete. The purpose of this study was to examine the physiological effects of resting (surviving crew would be confined to their bunks) exposure to mild hypoxia (16.75% O2), hypercapnia (2.5% CO2), cold (submarines cool to water temperature of about 4 deg C several hundred feet below the surface) and high humidity. The primary objective is to document average metabolic rate and CO2 production in crew. Estimates currently available are based on measurements thought inaccurate.

There was a mean decrease in total body water of 0.4kg during the study, as assessed by total body water (TBW) measured with deuterium oxide at the beginning and end of the study (Table 1). Water turn over was approximately 3 liters/day over the entire period, but was higher during the first 4 days of the study. Energy expenditure increased throughout the study, averaging 3559 kcal/d during the first 3 days, 3813 kcal/d from days 0-5, and 4476 kcal/d throughout the whole 7 days. Energy expenditure calculated using the 2 point and regression analysis gave very similar results for days 0-5 and days 0-7.

Table 1. Stable Isotope Data

	Total Body Water (kg)									
	Initial		**Final**	**Water Turnover (L/d)**		**Energy Expenditure (kcal/d)**				
S#	**O18**	**Deuter.**	**Deuter.**	**d 0-5**	**d 0-7**	**2 pt d 0-3**	**Regres. 0-5**	**2 point d 0-5**	**Regres. d 0-7**	**2 point d 0-7**
1	50.2	49.0	47.0	3.2	3.0	3184	3701	3725	4395	4368
3	44.9	44.0	43.8	3.3	2.9	2751	3163	3332	3968	4127
4	45.1	44.1	43.9	3.0	2.7	3550	3759	3726	4363	4398
5	42.4	42.0	42.4	2.6	2.5	2936	3479	3741	3936	4020
8	53.9	51.7	51.7	4.0	3.8	5373	4965	5026	5718	5634
Avg	47.3	46.2	45.8	3.2	3.0	3559	3813	3910	4476	4510
SD	4.6	4.0	3.7	0.5	0.5	1057	685	647	727	649

Body composition data was used to calculate body energy stores used for energy, which combined with dietary intake was used to estimate the average respiratory quotient to be used in calculating energy expenditure by the doubly labeled water method (Table 2). The average fat free mass and fat mass lost during the study was 0.92 kg and 0.63 kg. Assuming that 300 g of glycogen was utilized, and combined with the dietary intake, this gave an RQ of 0.82 and an energy equivalent of 5.794 (Table 3). However, the body stores used, or the estimated energy intake were significantly underestimated, as the Energy Expenditure obtained from this Intake/Balance method was 955 kcal/d lower than that obtained from the DLW method.

Table 2. Body composition

	Total Body Water								
	O18 difference	Hydration of Fat Free Mass		at Free Mass, kg			Fat Mass, kg		
	70.2%	70.2%	73.2%	Initial	Final	loss	Initial	Final	loss
1	0.0	50.3	52.4	71.59	69.69	1.90	22.11	21.41	0.70
3	-0.5	44.5	46.4	63.35	62.92	0.43	7.35	6.68	0.67
4	0.8	46.0	47.9	65.49	64.57	0.92	20.91	20.73	0.18
5	0.5	42.9	44.8	61.14	61.17	-0.03	20.16	19.63	0.53
7				73.60	72.93	0.67	21.00	19.97	1.03
8	-0.7	53.1	55.4	75.70	73.86	1.84	6.40	5.64	0.76
9				58.40	57.66	0.73	15.80	15.24	0.57
Avg	0.0	47.4	49.4	67.0	66.1	**0.92**	16.2	15.6	**0.63**

When an FQ was estimated based on the energy expenditure and body stores necessary to make up the difference, a lower FQ of 0.799 was obtained, but the energy equivalent for CO_2 was quite similar at 5.883. (For an in depth analysis of the FQ and Intake balance calculates see Table 3 in the 12th Quarterly report.) That the intake balance method would not give an accurate measure of energy expenditure over such a short time is not surprising, as the error in body composition and energy intake are quite high for these calculations. However, the major reason for this discrepancy was that the final body composition measurement was obtained 2 days after subjects resumed ad lib feeding.

We have also begun to receive samples form the Ft. Carson study of energy balance with the 10th SFG.

C. Key Research Accomplishments

- Conducted stable isotope measures during an Infantry Officer Training Course, conducted at Quantico during the winter. Major outcome variables examined were total daily energy expenditures, energy deficits and water intake and hydration status.

- Conducted stable isotope measures during an Infantry Officer Training Course, conducted at Quantico during the summer. Major outcome variables examined were total daily energy expenditures, energy deficits and water intake and hydration status.

- Carried out doubly labeled water studies during a 9-day simulation of a disabled submarine (DISSUB) with eight volunteer "survivors" conducted in the NATICK chamber facility in an Infantry Officer Training Course, conducted at Quantico. Major outcome variables examined were total daily energy expenditures, energy deficits and water intake and hydration status.

D. Reportable Outcomes

1. Tharion, W.J., C.J. Baker-Fulco, S. McGraw, W.K. Johnson, P. Niro, J.P. Warber, F.M. Kromer, R. Allen, C.M. Champagne, C. Falco, R.W. Hoyt, J.P. DeLany and L.L. Lesher. The effects of 60 days of tray ration consumption in marine combat engineers while deployed on Great Inagua Island, Bahamas. Chapter 6: Energy expenditure, water turnover and hydration status. W.J. Tharion, C.J. Baker-Fulco, R.W. Hoyt, and J.P. DeLany. *USARIEM Technical Report* T00-16 Natick, MA: U.S. Army Research Institute of Environmental Medicine, January 2000.

E. Conclusions

The average energy expenditure during the Winter Infantry Officer Training Course was quite high, 6472 ± 816 kcal/d. We expected somewhat elevated energy expenditure, as the weather was cold, soldiers were carrying around 100-150 lbs. and were getting very little sleep.

Average energy expenditure during the summer study under otherwise similar conditions was also high, but was considerably lower than during the winter (4107± 600 kcal/d).

The participants in the simulated disabled submarine survival study had a surprisingly high-energy expenditure, considering their low activity level. The exposure to hypercapnic, mildly hypoxic cold conditions appear to cause a significant increase in energy expenditure. The fluid balance of the subjects during this study did not appear to be dramatically affected over the course of the study, as the total body water lost by the end of the study was only 0.4 kg.

F. References

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III. Stress, Nutrition and Mental Performance

A. Introduction

The objective of this task is to identify diets or nutritional supplements that will improve the behavioral response to stress. All of the work involves the use of rodent models of stress with end-point behavioral measures that include anxiety, memory and feeding behavior. Two strategies have been employed the first was to use information published by others to identify potentially beneficial nutritional interventions and then to test these in our rodent models. This approach has proved largely unsuccessful. The second strategy is to try and identify which central and physiological pathways mediate the stress-induced behaviors with the expectation that this information will provide a basis for identification of dietary factors that could prevent activation of these pathways. The varied skills of the research staff working on this project allows us to evaluate the behavioral response to stress from the whole animal down to the molecular level. Using this approach we have successfully identifies two potential markers for stress responsiveness and have started to clarify the biochemical basis of the some of the behavioral responses observed in our stressed rats and mice.

B. Body

Identification of a Natural Urocortin Antisense RNA in Rat Tissues
Mingxia Shi

Introduction

Urocortin (UCN) is a neuropeptide related to corticotrophin releasing factor (CRF) in mammals (Vaughan et al., 1995). There is increasing evidence that UCN may be involved in central appetite control, and in behavioral and endocrine responses to stress (Spina et al, 1996; Turnbull et al., 1999). Rat urocortin was originally cloned from a midbrain cDNA library. We recently identified the existence of a naturally occurring rat UCN antisense RNA in rat tissues. Natural antisense RNAs are endogenous transcripts that exhibit complementary sequences to their mRNA counterparts. Many studies suggest that antisense RNA transcripts are involved in the control of various biological functions including regulation of their corresponding sense mRNAs. This regulation may be exerted at many levels of gene expression (transcription, maturation, transport, stability and translation). Some antisense RNAs may also encode for proteins. In last year's annual report, we described the results from a ribonuclease protection

assay (RPA), which provided a significant amount of sequence for the central portion of the antisense mRNA. However, the remaining 5' and 3' sequences were needed to determine whether the sense and anti-sense mRNA had identical, but reversed sequences. This year we identified the full-length sequence of rat UCN antisense RNA, which provided further information on the potential function of UCN antisense mRNA.

Materials and Methods

Total RNA was extracted from the tissues of rats that had been exposed to 3 hours of restraint stress. We attempted to identify the 3' and 5' ends of UCN antisense mRNA by RACE (rapid amplification of cDNA ends) reactions and by primer extension reactions. Therefore, additional riboprobes for RPAs were developed using sequences designed to detect the 3' and 5'ends of anti-sense mRNA, assuming identical, but reversed, sequence to sense RNA.

RT-PCR was conducted to amplify the 5' and 3'-end of UCN cDNA using two pairs of primers (UCN3/UCN4, UCN5/UCN6), described in Table 1. The PCR product of the predicted size was cloned into PCRII by TA cloning (Invitrogen). The inserted sequence was confirmed and the orientation was determined by sequence analysis (Gene Lab). Radiolabeled rat UCN sense riboprobes were synthesized in vitro using T7 RNA polymerase from three linearized cloned UCN templates of 280 bp (5'-end), 258 bp (3'-end) and 251 bp (middle segment) in length, respectively. The RPA was carried out using a kit from Ambion. Rat β-actin antisense riboprobe was used as an internal control. Full-length riboprobes were gel-purified and hybridized with 10 μg total RNA. Protected fragments were separated on a denaturing 5% polyacrylamide, 8M urea gel. The dried gel was finally exposed to a Phosphoimaging screen overnight.

Results and Discussion

As shown in Figure 1 (see Appendix), all three UCN sense riboprobes, corresponding to 5', middle and 3' regions of UCN RNA, detected protected signals in heart and muscle, but no protected fragment was detected in midbrain. This result further confirms the existence of a naturally occurring UCN antisense RNA, rather than another sequence with only partial homology to UCN. Thus, it is most likely that the rat natural antisense UCN transcript has full-length sequence complementary to the UCN sense mRNA. The sense and antisense RNA may be transcribed from opposite strands of the same UCN locus (cis-encoded antisense) and display perfect complementarity. The selective tissue distribution of the UCN antisense RNA demonstrated in present study is consistent with our previous results. In contrast, we have demonstrated that the level of UCN sense mRNA is high in midbrain but undetectable in heart and muscle. An inverse relationship between levels of accumulation of sense and antisense messengers has also been documented in many other cases, which suggests the regulation of sense expression by antisense transcripts. The antisense UCN transcription might impede sense UCN transcription. UCN antisense RNA may hybridize to sense RNA in vivo to modify, destabilize and down regulate sense expression due to its full-length overlapping with sense mRNA. As the two transcripts are expressed together in the hypothalamus but not midbrain or

heart, this type of regulation would be limited to specific tissues. An open reading frame was not found in UCN antisense RNA, so it is unlikely to produce a protein of its own.

Table 1: Primers used for PCR and development of riboprobes

Primer	Sequence	Location according to U33935
rUCN-P1	5'-GGCGAATGTGGTCCAGGA	153-170
rUCN-P2	5'-TGATGCGGTTCTGCTGTGC	389-403
rUCN-P3	5'-ATCCAGTCAGAGTGTTCAG	560-542
rUCN-P4	5'-CGCGCACTCCTCTTGCTGTT	220-239
rUCN-P5	5'-CTGAGCCAGCTCCGGTTGTG	492-511
rUCN-P6	5'-GCGGCCGCTCTCCATCTTG	1-19
rUCN-P7	5'-CTGCAGGCTCAGATCCCGC	262-280
RUCN-P8	5'-CCCGCCGTTGTCCATCGAC	303-321

Changes In Fat Glucose Transport And β-Adrenergic Receptor During The Post Stress Period In Rats Exposed To Repeated Restraint Stress

Jun Zhou, Mingxia Shi, Sonyja Thomas, Tiffany Mitchell, Ruth Harris

Introduction

In previous studies, we have found that 3 hours of restraint stress for 3 consecutive days causes a temporary inhibition of food intake and a chronic suppression of body weight in rats (Harris et al., 1998; Zhou et al., 1999), and the response is exaggerated by a high fat diet (Harris et al., 1998). Carcass analysis shows that restrained rats lose only lean body mass during stress, but by Day Five after the last restraint stress, the weight difference between control and stressed rats is composed of both lean and fat tissue. We have investigated the mechanisms that may be responsible for these body weight and composition changes and preliminary data indicated that glucose transport was decreased and β-adrenergic receptor number increased in fat tissue from restrained rats, measured 24 hours after the last restraint stress. The objective of the present study was to determine whether these changes were still present five days after the end of restraint, to clarify their contribution to changes in body composition observed during the post stress period in restrained rats.

Materials and Methods

Thirty-two adult male Sprague-Dawley rats, weighing 350g, were housed in individual wire mesh cages. They were fed high fat diet (40% kcal fat) for at least 10 days before being divided into three weight matched groups: Control, Restraint-1 (RS-1), and Restraint-5 (RS-5). Body weights and food intakes were recorded daily.

For repeated restraint rats were placed in Perspex restraining tubes (Plas Labs, Lansing, MI) for three hours in the morning for three consecutive days. The control rats were moved to the same room as the restrained rats and did not have access to food or water for the period of restraint. The rats in Restraint-1 group were killed one day after the last restraint stress and the rats in Restraint-5 group were killed five days after the last restraint stress. Half of the rats in the control group were killed one day after the last restraint stress and the other half of the control rats were killed five days after the last restraint stress. Prior to decapitation, rats were food deprived for 2 hours and then epididymal fat was dissected for measurement of glucose transport and β-adrenergic receptor number.

Adipocytes were isolated by the method of Rodbell (1964). The digested cells were filtered though a 125mm nylon mesh, washed three times in Krebs buffer, 0.1mM glucose, 2% BSA (pH 7.45) and re-suspended in an appropriate volume of wash buffer and held at 37°C for 20~30 minutes before use. Glucose uptake was measured in basal and insulin stimulated conditions (0, 0.8 mU insulin/ml). One ml of each cell suspension was added to 2 ml wash buffer containing 0.1 μCi/ml ^{14}C-mannitol and incubated for 30 minutes at 37°C with shaking. Cell number was determined by fixing an equivalent aliquot in osmium tetroxide and counting by Coulter Counter (Coulter Electronics Inc., FL). Then 0.2 mM 2-DG, 1.0 mCi/mM ^{3}H 2-DG in 50 μl volume was added and the sample was incubated for exactly 2 minutes. Triplicate 200 μl aliquots of the sample were transferred to vials containing 100ul phthalic acid dinonyl ester and immediately centrifuged to separate cells from media. All incubation conditions were carried out in duplicate. The cell fraction was counted for 2-DG incorporation and corrected for extracellular fluid volume, indicated by ^{14}C-mannitol. Results were expressed as nmol glucose incorporated per 10^6 cells per 2 min.

For measurement of adrenergic receptor number, dissected epididymal fat was immediately homogenized in cold homogenization buffer (Krebs bicarbonate buffer pH 7.45, 10μg/ml aprotinin, 5μg/ml leupeptin, 1mM phenylmethlsulfonyl fluoride), centrifuged at 1000xg (3000rpm) for 10 minutes at 4°C, and the supernatant was further centrifuged at 13500xg for 15 minutes at 4°C. The final pellet was dissolved in homogenization buffer and protein concentration determines (BCA kit; PIERCE, Rockford, IL). The final protein concentration was adjusted to 200μg/ml. For maximum β-adrenergic receptor binding activity, 50ul freshly isolated plasma membrane was incubated at 37°C for 30 minutes in 0.3ml homogenization buffer with 200nM ^{3}H-DHA. For nonspecific binding, a saturating concentration of propranolol

(5mM/ml) was present in the incubation buffer. All incubation were carried out in duplicate, the reactions were stopped by adding 1.0ml ice cold homogenizing buffer to the tubes and placing the tubes on ice. Membranes were collected by vacuum filtration through a 0.45μm nitrocellulose filter (Millipore, Bedford, Massachusetts) and washed with 10 ml cold Krebs buffer. The filter was dissolved in 1ml of ethylene glycol monoethyl ether (Sigma. Chemical Co. St. Louis, MO) and bound ^{3}H-DHA determined by scintillation counting. The specific binding was determined by subtracting nonspecific binding from maximum binding.

Repeated measurements of variance were used for statistical analysis of adipocyte glucose transport with insulin concentration as the repeated measure. One way ANOVA with post-hoc Tukeys' test was used for β-adrenergic receptor antagonist binding. The SAS system version 6.12 was used for computations. Data are presented as means ± SEM.

Results

As we have reported previously, repeated restraint caused rats to lose body weight and reduce their food intake (data not shown). The results from adipocyte glucose transport are illustrated on Figure 2 (see Appendix). Insulin significantly stimulated adipocyte glucose transport ($P=0.01$). One day after the last restraint stress, adipocyte glucose transport was significantly reduced in fat from RS-1 rats, compared with controls ($P<0.05$). The same measurements, carried out five days after the last restraint stress, indicated that glucose transport was in cells from RS-5 rats was not significantly different from that of either control or RS-1 rats. Maximum β-adrenergic receptor binding is also shown in Figure 2 (see Appendix). The RS-1 group rats had a significantly higher maximum binding activity then control or RS-5 rats ($P<0.05$), which indicates that their adipose plasma membrane β-adrenergic receptor number was up-regulated. There was no difference between control and RS-5 groups.

Discussion

The objective of this experiment was to investigate mechanisms that contribute to changes in body composition of restrained rats during the post-stress period. Our previous results show that stressed rats lose only lean body mass during stress but that they have lost both lean and fat body mass on Day Five after stress (Harris et al., 1998; Zhou et al, 1999). The changes in body fat content could result from changes in lipolysis and/or lipogenesis. It is well known that increased sympathetic tone causes lipolysis and decreases nutrient supply, inhibiting lipogenesis (Hoffsted et al., 1997). β-adrenergic receptor binding is an important determinant of sympathetic activity. The results from these experiments show that maximum β-adrenergic binding activity increased in restrained rats at the beginning of the post-stress period and then returned towards control levels by Day Five after stress. Adipocyte glucose uptake in restraint rats also showed a transient decrease one day after stress and but had returned towards control levels by Day Five. Therefore, we can conclude that the loss of body fat mass in the early post stress period is caused by both an increase in β-adrenergic receptor number and an inhibition of glucose transport in adipose tissue, which would increase lipolysis and decrease lipogenesis.

However, lipolysis and lipogenesis are regulated my many factors and further studies are needed to fully elucidate the chronic metabolic response to restraint stress.

The Effect of Food Availability and Timing of Stress on Weight Loss In Rats

Ruth Harris, Tiffany Mitchell, Sadie Herbert and Jun Zhou

Introduction

We have previously found that rats that have been exposed to repeated restraint (3 hours of restraint on three consecutive days) lose weight during the stress and do not return to the weight of control rats once the stress has ended. The weight loss is associated with an inhibition of food intake on the days of restraint and a failure to compensate by overeating during that days following the end of restraint (Harris et al., 1998). The amount of weight lost by the rats appeared to be determined by the time of day that the rats were stressed. Those stressed at the end of the light period, or at the beginning of the dark period, lost less weight than those stressed at the start of the light period (Rybkin et al., 1997).

The objective of this experiment was to determine whether the amount of weight loss caused by repeated restraint stress was determined by a relationship between the time that stress was applied and the normal feeding time of the rat. Rats are nocturnal feeders and we have found that the majority of the change in food intake of stressed rats is due to inhibition of intake at the start of the dark period (Harris et al., 1998). In this experiment we had rats that were given ad libitum access to food 24 hours/day, some that were fed only at night and others that were fed only during the day. Within each of these feeding treatments we examined the effect of repeated restraint on body weight when the stress was applied either at the start of the light period or at the start of the dark period.

Methods

Ninety-six 300 g male Sprague Dawley rats were housed individually and were adapted to a high fat diet containing 40% kcal fat, 16% kcal protein for 7 days prior to the start of the experiment. Daily body weights were recorded and the rats were divided into three weight-matched groups. One group continued to have free access to food for 24 hours a day (Ad Lib), the second group was only offered food during the light period (Light fed) and the final group was given food only during the dark period (Dark Fed). Red lights were on for 24 hours a day in the homeroom of the rats to facilitate data collection at the start of the dark period. Daily food intakes and body weights were recorded while the rats adapted to the feeding regime. All of the measurements were taken at the end of the 12 hour period that the rats had eaten, i.e. Ad lib and dark fed rats were weighed at the start of the light period and light fed rats were weighed at the start of the dark period. After 2 weeks, the rats within each feeding treatment were then subdivided into four weight matched groups of 8 rats: light stressed, light control, dark stressed and dark control. The stressed rats were subjected to 3 hours of restraint on each of 3 days, as described above. Light stressed rats were restrained from 8.00 a.m. to 11.00 a.m. and dark stressed were restrained 7.30 p.m. to 10.30 p.m. The dark stress was carried out in a room

illuminated only with red light. A tail blood sample was collected from each rat after 1 hour of the first restraint stress.

The rats were allowed to recover from the stress for 5 days and were then killed for measurement of serum corticosterone and fat pads were weighed. Epididymal fat was frozen for measurement of β3-adrenergic receptor by Western blot. The light fed rats were killed at the start of the dark period (8.00 – 9.00 p.m.) and the Ad lib and Dark fed rats were killed at the start of the light period (8.00 a.m. to 11.00 a.m.).

Results

This study was completed only recently, therefore, the results have not been analyzed statistically and interpretation of the data is qualitative. The body weights of the rats are shown in Figure 3 (see Appendix). Ad lib and dark fed rats lost weight in response to restraint stress, regardless of the time of day that stress was applied. Rats fed during the light period did not lose weight, regardless of the time of day that stress was applied. Food intakes of the rats are shown in Figure 4 (see Appendix). Stress had the greatest effect on the food intake of Dark fed rats and had no effect on the intake of Light fed animals. The time of day that stress was applied had no effect on the response in any of the three feeding treatments. Therefore, the weight loss was not related to serum corticosterone concentration, as the diurnal rhythm of release had not been changed by the feeding schedule and was the same in all three groups of rats. This is demonstrated in Figure 5 (see Appendix), which shows corticosterone measures made both during restraint and at the end of the experiment. The light fed rats were killed at the start of the dark period and their corticosterone was higher than that of the two other feeding treatments, as would be expected based on a normal circadian rhythm for glucocorticoids.

Discussion

These results demonstrate that the feeding schedule of the rats does influence stress-induced weight loss, however, the response is independent of glucocorticoid release. We have previously found that there is a substantial increase in the β-adrenergic receptor number of rats exposed to repeated restraint (see above). Therefore, it is possible that a difference in activation of the central catecholamine, or peripheral sympathetic nervous system, is modified by feeding time and this, in turn, changes the energetic response to stress.

Protective Effect of Insulin on Dopaminergic Neurons

Patrick Awenowicz and Xiaochun Xi

Introduction

There is an increasing amount of evidence that insulin in the brain may have neuroprotective effects. There is a negative correlation between insulin levels and the risk for Parkinson's and Alzheimer's disease (Mattson et al., 1999). Parkinson's disease is characterized by degeneration of the midbrain dopaminergic system, specifically by a loss of dopamine producing neurons in the substantia nigra pars compacta (Snpc). Additionally,

immunohistochemical and *in situ* hybridization studies have revealed an abundance of insulin receptors within the Snpc (Takahashi et al., 1996). Post-mortem examination of the Snpc of Parkinson patients has revealed an absence of insulin receptors, in addition to the loss of dopaminergic neurons (Takahashi et al., 1996). The following project was designed to examine the potential role of insulin on the midbrain dopaminergic system. The first part of this project was intended to examine the effects of peripherally administered insulin on 6-hydroxy-dopamine (6-OHDA) induced lesions of the Snpc. The ultimate goal was to determine if insulin treatment could promote neuronal survival during and following neurotoxic lesioning.

Materials and Methods

Twenty-four male Sprague Dawley rats, weighing 250g, were divided into 4 groups of 6: 1- Insulin pump/6-OHDA; 2- Insulin pump/saline; 3- Saline pump/6-OHDA; 4- Saline pump/saline. Following anesthesia with isofluorane, Alzet osmotic mini-pumps (0.5 ul/hr), filled with insulin (0.250 U/ul) or saline, were placed subcutaneously in the scruff of the neck. On the third day following minipump implant, blood and CSF insulin levels were measured.

Three days following mini-pump implant, the rats were anaesthetized with ketamine and xylazine, the head shaven and the rat placed in a stereotaxic frame. A skin incision was made, the wound cleaned and the skull surface cleared of membrane. Using a power drill a hole was made over the site of injection of the catecholamine neurotoxin 6-OHDA. Stereotaxic coordinates for the substantia nigra were obtained from Paxinos and Watson atlas, (B: 5.3 mm, M-L: 2.4 mm, D-V: 7.7 mm). An injection of 6-OHDA (80 ug in 4 ul saline) or saline was made into the substantia nigra with a Hamilton syringe over 8 minutes at a rate of 0.5 ul/min. Seven days after the lesion, dopamine concentrations in the Snpc and frontal cortex were determined by HPLC in 16 of the rats. The remainder of the rats, CSF was collected to determine whether the peripherally administered insulin increased central insulin levels. Following this they were transcardially perfused with 4% paraformaldehyde. The brains of these rats were processed for immunohistochemistry with an antibody for tyrosine hydroxylase.

Results

Peripheral administration of human insulin significantly increased CSF insulin levels compared to saline treated controls. Immunohistochemistry for tyrosine hydroxylase revealed the expected dense labeling in the Snpc as well as dense fiber labeling in the frontal cortex. Following lesioning with 6-OHDA the level of tyrosine hydroxylase was greatly reduced. The study on the effects of insulin on 6-OHDA lesions has not been completed.

Discussion

These results demonstrate that peripherally administered insulin increased CSF insulin concentration, so that peripheral insulin should provide a protective effect centrally. The immunohistochemistry confirmed that the 6-OHDA caused significant lesioning and that the tyrosine hydroxylase antibody was specific. As Patrick Awenowicz ahs left the group, studies

examining the protective effect of insulin against damage to dopaminergic neurons will not be completed.

The Melanocortin System as a Marker for Stress Responsiveness

Jun Zhou and Mingxia Shi

Stress stimulates the secretion of many hormones, including ACTH. The precursor of ACTH is proopiomelanocortin (POMC) from which other peptides, including α-melanocyte stimulating hormone (αMSH), are also derived. αMSH activates melanocortin receptors (MC-R) of which five subtypes have been reported so far (Fisher et al., 1999; Kiefer et al., 1998). MC-R1 is expressed in the skin and regulates skin color. MC-R2 is the ACTH receptor, expressed in the adrenal cortex and mediates ACTH stimulated corticosterone synthesis. MC-R3 and MC-R4 are both expressed on the brain and are involved in the regulation of food intake. MC-R5 is expressed in exocrine glands and has been implicated in the control of lipid and pheromone production.

Agouti protein is a 132 amino acid paracrine factor encoded by the mouse agouti gene (Sipos and van Heijne, 1993; Spiegelman and Flier, 1996) which is an antagonist of all subtypes of melanocortin receptors making it likely that agouti protein is involved in the stress response. Mice overexpressing agouti protein have a yellow coat color, moderate obesity, type II diabetes, and increased somatic growth (Baas et al, 1976; Weigle et al, 1999). There are no reports describing the stress responses of mice overexpressing agouti protein. The objective of the present study was to determine whether mice overexpressing agouti protein exhibit an exaggerated stress response, using different behavioral tests for measurement of anxiety and to identify which stress activated pathway, the HPA axis or the catecholaminergic system was disrupted by agouti.

The results of the first experiment demonstrated that mice over-expressing agouti protein were more responsive to stress than wild type mice. As agouti protein antagonizes all melanocortin receptors, it is reasonable to hypothesize that one of the melanocortin receptors normally acts to dampen the stress response. However, the increased stress response could result from developmental compensation in transgenic mice overexpressing agouti protein, rather representing involvement of the melanocortin system under normal conditions. To clarify the importance of acute versus chronic antagonism of the melanocortin system, we originally planned to inject agouti protein into wild type mice and measure their stress response. If the wild type receiving agouti protein showed the same stress response as those over expressing agouti protein, then it is likely that the melanocortin system modulates the stress response. However, agouti protein was no longer commercially available. As an alternative strategy, we determined whether a melanocortin receptor agonist, α-Melanocyte stimulating hormone (α-MSH), inhibited the stress response. We pretreated wild type mice with α-MSH to measure their endocrine and behavior response to restraint stress.

Materials and Methods

Experiment 1: Stress Responsiveness In Mice Overexpressing Agouti Protein

Twelve-week old male, wild type and heterozygote transgenic mice, in which a β-actin promoter was used to induce ectopic expression of agouti (BAP), were provided by Dr Randall Mynatt, PBRC. The mice were housed individually and had free access to the water and diet throughout the experiment. All mice were handled and weighed daily for 5 days before dividing each genotype into two groups: control and restraint stress. Thus, there were four groups of mice: (1) BAP control. (2) BAP restraint. (3) Wild type control. (4) Wild type restraint.

Body weights were recorded daily and after five days of baseline, restrained mice were placed in plastic restraining tubes for 12 minutes. Immediately after restraint stress, a behavioral test was started. Three behavioral experiments were carried out: Defensive withdrawal, Light-dark box and Elevated Plus Maze. The interval between each exposure to stress and the behavioral tests was 6 days. The defensive withdrawal behavioral apparatus consisted of a 0.5m square open field with a white floor. A cylindrical chamber (length 10 cm, diameter 6.5 cm), open at one end, was secured to the floor lengthwise next to one wall 20cm away from the corner. To start the test, a mouse was placed in the chamber and various behaviors were observed during the 5 min session by Video Tracking, Motion Analysis & Behavior Recognition System (Noldus Information Technology, VA). The light-dark box consisted of a polyvinylchloride box divided into two chambers (23.5×23.5×23.5in.). One side had black walls, floor and ceiling and the other side had white walls and floor and was illuminated by a 75-watt bulb placed 3 in. above the box. The dark and light compartments were connected by a semicircular hole (9cm high and 5cm wide). Each mouse was tested for 5 minutes. The mouse was introduced into the dark side of the box and the following behaviors were recorded: (1) Latency first to escape from dark box. (2) Number of exits from the dark box. (3) Time spent in the light box. (4) Mean duration of visits to the light box. The elevated plus maze consisted of two open arms (10×48cm.) and two closed arms (10×48 in.) angled at 90° to each other and extending from a central platform (10×10 in.). The apparatus was elevated 50cm above the floor. Each mouse was placed on the center platform facing an open arm. The test period was 5 minutes and the following behaviors were recorded: (1) Number of entries into the closed arm. (2) Time spent in the closed arm. (3) Number of entries into the open arm. (4) Time spent in the open arm.

The experiment ended 10 days after the last behavioral test. Immediately after 30 minutes of restraint, the mice were decapitated and blood was collected. The brain was frozen on dry ice for catecholamine determination. Thymus and adrenal glands were weighed. Each hypothalamus was dissected and sonicated in 400ul 1% trifluoroacetic acid. An equal amount of 1% trifluoroacetic acid was added and samples were centrifuged at 10000g for 20 minutes at 4°C. The CRF was extracted using C-18 Sep-columns according to manufacturers instructions (Peninsula Laboratories, Inc., CA). The final extract was dissolved in 190ul assay buffer and 50ul of extract was used to determine CRF using an EIA kit (Peninsula Laboratories, Inc. CA).

Protein concentration of each sample was determined by BCA protein assay kit (PIERCE, Illinois). After being dissected and weighed, the amygdala was sonicated in 150ul 0.1M perchloric acid, 0.1mM EDTA. The samples were centrifuged twice at 14,000g, 4°C for 15 minutes and then separated by HPLC (ESA Inc., Massachusetts) to determine catecholamine concentration. The mobile phase was composed of 75 mM sodium phosphate, pH 3.1, 1.4 mM 1-octanesulfonic acid, 0.1 mM EDTA and 20% acetonitrile at flow rate 0.3ml/minutes. The electrical potential applied to analytical cell (5014B Microdialysis cell. ESA INC., Chelmsford, MA) was –0.04 volts for electrode 1 and +0.34 volts for electrode 2. The catecholamines and metabolites detected were: norepinephrine (NE) and its metabolite MHPG, serotonin (5-HT) and its metabolite 5-HIAA, Dopamine (DA) and its metabolites DOPAC and HVA. Sample protein concentration was determined using the BCA protein assay kit. All catecholamine concentrations were expressed as ng/ug tissue protein

Experiment 2: Stress Responsiveness Of Mice Injected With α-MSH

In a pilot study groups of 10 mice were injected i.p. with increasing doses of α-MSH, to determine the effective dose for stimulating corticosterone release through agonism of the ACTH receptor. As shown in Figure 7 (see Appendix), restraint stress increased plasma corticosterone concentrations and 160 ug/mouse αMSH injection significantly exaggerated this effect ($P<0.05$).

Forty, male, C57BL/6J mice were housed individually with free access to water and chow and were adapted to handling before the experiment started. They were divided into two groups: Saline injected (0.2 ml i.p.) and α–MSH injected (160 ug in 0.2 ml saline, i.p.). Thirty minutes later, mice in each group were subdivided into restrained (Saline rs or α–MSH rs) and non-restrained groups (Saline c or MSH c). Immediately after 12 minutes of restraint stress, anxiety behavior was tested in the light-dark box. Three days later, daily measures of food intake and body weight were initiated. After 10 days of baseline measures, the mice were subjected to repeated restraint stress. For repeated restraint, the mice received the same injections of either saline or α-MSH and remained in the previously assigned four treatment groups. Restraint was extended to two hours per day and it was repeated for three days with the mice being injected 30 minutes before restraint on each of the 3 days. Thirty minutes after starting the first restraint stress, a small amount of blood was collected from each mouse by tail bleeding to measure corticosterone. At the end of the last restraint stress, rectal temperatures were measured. The measurements of food intake and body weight were continued for another ten days after the last restraint stress.

Statistical Analysis

The non-numeric data was analyzed by two-way ANOVA with multiple comparisons (Tukey) method. The numeric data was analyzed by a non-parametric method (Chi-square). Food intakes and body weight data were analyzed by repeated measurements of analysis with day as the repeated measure. SAS 6.12 was used for calculation. Data are presented as mean $\pm$ SE.

Results – Experiment 1

Body weight changes are shown on Figure 6 (see Appendix). Restrained mice of both genotypes lost more weight than their controls following stress and behavioral tests, but there was no statistically significant difference in the change of weight for either genotype. The results of the defensive withdrawal test are summarized in Table 2. Restraint stress tended to reduced the distance traveled ($P=0.05$) and decreased velocity ($P<0.05$), but the multiple comparison did not show any significant difference between genotypes or between individual groups. Time spent rearing was reduced and grooming increased in restrained mice ($P<0.01$), and wild type restrained mice spent less time rearing and more time grooming than their controls ($P< 0.05$). Time spent out of the chamber was reduced in restrained mice of both genotypes ($P<0.05$) but there was no significant difference between groups by multiple comparison. There was no effect of stress or genotype on any of the other measurements.

Table 2. Results from the Defensive Withdrawal Test

	BAP		**Wild Type**	
	Control	*Restrained*	*Control*	*Restrained*
Distance moved (cm)	2007 ± 241	1546 ± 276	2172 ± 206	1546 ± 276
Velocity (cm/s)	6.9 ± 0.8	5.4 ± 0.9*	7.4 ± 0.7	5.7 ± 0.8*
Number of rears	24 ± 3	13 ± 4	23 ± 2	12 ± 3
Duration of rears (sec)	50 ± 9	12 ± 3	48 ± 6	22 ± 5*
Grooming number	1 ± 0	4 ± 1	2 ± 0	5 ± 2
Grooming time (sec)	2 ± 1	12 ± 4	6 ± 1	30 ± 9 *
Exits from chamber	6 ± 1	3 ± 1	5 ± 1	3 ± 1
Time out of chamber (sec)	254 ± 13	186 ± 35*	258 ± 12	207 ± 26*
Entries into center zone	10 ± 2	7 ± 3	11 ± 2	10 ± 2
Time in center zone time (sec)	15 ± 2	10 ± 4	18 ± 2	20 ± 5

Data are means ± sem for groups of 10 mice. An asterisk indicates a significant difference ($P< 0.05$) compared with controls, determined by two way ANOVA analysis.

The results from the light dark box are summarized in Table 3. Restrained BAP mice showed a significant increase in the latency to escape from the dark box and a reduced number of exits from the dark box ($P<0.01$) compared with their controls. In the same experimental conditions, the restraint wild type mice did not show any differences in behavior compared with their controls. There was no effect of stress on time or mean time interval spent in the light box for either genotype.

Table 3: Results from the Light-Dark Box Test

	BAP		**Wild Type**	
	Control	*Restrained*	*Control*	*Restrained*
Latency to escape dark box (sec)	32 ± 4	68 ± 13	43 ± 5	56 ± 10
Exits from dark box	7 ± 1	4 ± 1	8 ± 1	8 ± 1
Time spent in light box (sec)	96 ± 6	90 ± 26	88 ± 9	103 ± 12
Mean interval in light box (sec)	14 ± 2	22 ± 7	11 ± 1	15 ± 2

Data are means ± sem for groups of 10 mice.

Results from the Elevated plus-maze are shown in Table 4. Restrained BAP mice significantly reduced the number of entries into the closed arms compared with their controls ($P<0.05$) and both restrained and control BAP mice spent more total time in the closed arms than did wild type restrained mice. Wild type restraint mice significantly reduced the time spent in closed arms compared with their controls ($P<0.05$), made more entries into the open arms than either controls or restrained BAP mice ($P<0.05$) and spent more time in the open arms than controls ($P<0.05$).

Table 4: Results from Elevated Plus Maze

	BAP		**Wild Type**	
	Control	*Restrained*	*Control*	*Restrained*
Entries into closed arms	14 ± 1	10 ± 1 *	13 ± 1	12 ± 1
Time spent in closed arms (sec)	136 ± 14	133 ± 19	140 ± 14	90 ± 7 *
Entries into open arms	12 ± 2	10 ± 1	10 ± 1	14 ± 1
Time spent in open arms (sec)	93 ± 16	98 ± 10	94 ± 15	154 ± 10 * #

Data are means ± sem for groups of 10 mice. * $P<0.05$ compare with controls, # $P<0.05$ compared with BAP restraint mice

There was no effect of genotype or treatment on adrenal or thymus weight (data not shown). Values for hypothalamic CRF content, measured after 30 minutes' restraint, are shown in Figure 7 (see Appendix). To facilitate understanding of the HPA axis response to restraint stress, plasma ACTH and corticosterone concentrations are also presented. The wild type mice had a normal stress response, represented by increased hypothalamic CRF content and a statistically significant increase in plasma ACTH and corticosterone levels. However, the BAP mice did not show increased levels of CRF or ACTH, although stress-induced corticosterone was higher than in stressed wild type mice. Frontal cortex catecholamine concentrations are illustrated in Figure 8 (see Appendix). Norepinephrine turnover was increased in BAP compared with wild type mice ($P=0.07$). Amygdala catecholamine concentrations for the different groups of mice are illustrated in Figure 9 (see Appendix). The norepinephrine metabolite, MHPG, and serotonin metabolite, 5-HIAA, were increased in restrained mice ($P=0.07$) but there was no

significant effect of genotype on the response. There were no significant effects of genotype or stress on the concentrations of other proteins measured in either brain area.

Results – Experiment 2

There was no effect of αMSH on any of behavioral measures made in the light-dark box test (data not shown). Serum corticosterone, measured after the first 30 minutes' restraint stress, is illustrated in Figure 10 (see Appendix). α-MSH treated mice had higher levels of corticosterone than saline injected animals ($P=0.06$) but there was no significant effect of restraint and no interaction between α-MSH and stress. Rectal temperatures, measured at the end of the last repeated restraint stress, are shown in Figure 11 (see Appendix). There was no significant effect of α-MSH injection, however, restraint stress significantly reduced body temperature, compared with non-restrained mice, in both saline and α-MSH injected groups. ($P<0.01$) There was no significant interaction between injection and stress.

Change in body weight after repeated restraint is shown in Figure 12 (see Appendix). Repeated restraint caused mice to lose weight on the days that they were stressed. During the post-stress period, the body weight of restrained mice remained lower than that of non-restrained mice, up to seven days after stress had ended. There was no effect of α-MSH on weight loss in stressed mice and it did not effect the body weight of non-restrained controls. Food intake during repeated restraint is shown in Figure 13 (see Appendix). Restraint significantly inhibited food intake but once stress ended, the food intake of restrained mice returned to control levels. There was no effect of α-MSH on food intake of either stressed or control mice.

Discussion

The results from Experiment 1 indicate that mice overexpressing agouti protein are more responsive to stress than their wild-type controls. The increased responsiveness is measurable both as increased anxiety behavior and as an exaggerated neuropeptide response. The results from the second experiment suggest that the increased responsiveness is secondary to a developmental change that occurs in transgenic mice experiencing chronic blockade of the melanocortin system, as acute activation of melanocortin receptors had no effect on the behavioral or endocrine response to stress.

Under conditions of stress, two of the pathways that are activated are the sympathetic nervous system and the HPA axis, both of which can stimulate plasma corticosterone. Our data suggests that the HPA axis is responsible for the stress-induced increase in corticosterone in wild type mice, whereas activation of the sympathetic nervous system, represented by increased norepinephrine turnover, is likely to be responsible for the increased corticosterone in BAP mice. In BAP mice, the CRF stimulation of ACTH secretion was blunted. Agouti protein blocks all melanocortin receptors, including MC-R2, an ACTH receptor, however, our observations indicate changes in ACTH secretion. One possible explanation for this is that corticosterone downregulates hypothalamic CRF and stimulates pituitary CRF-binding protein, which would suppress ACTH secretion.

The reason for this increased anxiety in BAP mice is not clear, but increased HPA axis activity is unlikely to be responsible, since ACTH in BAP mice was not increased by 30 minutes of restraint stress, while ACTH in wild type stress mice increased substantially under the same conditions. Yaswen et al. (1999) reported that POMC deficient mice had lower ACTH but higher plasma catecholamine concentrations than their wild type controls. The measurements of catecholamine turnover in frontal cortex of mice Experiment 1 suggest that this is also true for BAP mice. Therefore, the increased anxiety response to stress might result from activation of the catecholamine system, rather than HPA axis activation. In contrast to the frontal cortex, there were no difference in caetecholamine content or turnover in the amygdala, an area of the brain that has been associated with emotion, indicating that the neurological response to stress in BAP mice is site specific.

Further studies are needed to clarify which of the melanocortin receptors is responsible for the increased stress-responsiveness in BAP mice and to confirm that acute blockade of specific receptors does not modify the behavioral or physiological response to stress.

Effects of Leptin on The Inflammatory Response to Endotoxin in db/+ and db/db Mice

Ruth B.S. Harris, Mary D. Boudreau, Dianne Dunning, Tiffany Mitchell

Introduction

Leptin, produced primarily by adipose tissue, has the tertiary structure of a long chain helical cytokine (Zhang et al., 1997) and it's receptor belongs to the family of Class I cytokine receptors (Tartaglia et al., 1995). Evidence for involvement of leptin in immune function is provided by impairment of T-cell immunity in ob/ob mice, which do not produce leptin, and in db/db mice, which have a mutated long-form leptin receptor (Chandra, 1980; Fernandes et al., 1978). In both genotypes, there is a diminished in vivo response to cell mediated and humoral immune challenges, but spleen cells respond normally to mitogens in vitro, indicating an environmental deficiency in the immune response (Chandra, 1980; Fernandes et al., 1978). Leptin may also provide the link between immunosuppression and malnutrition as it reverses starvation induced suppression of cellular immune responses (Lord et al., 1998).

In the cell-mediated immune response, antigens are presented to T cells, which release lymphokines that activate macrophages. T cells, rather than antigen presenting cells, respond to leptin, promoting production of proinflammatory cytokines (Lord et al., 1998). In addition to this indirect activation, leptin directly stimulates macrophage phagocytic activity (Gainsford et al., 1996; Loffreda et al., 1998) and enhances endotoxin-induced production of TNFα , IL-6 and IL-12 by a post-transcriptional mechanism (Loffreda et al., 1998). In vivo, leptin blunts the increased sensitivity of ob/ob mice to the endotoxin lipopolysaccharide (LPS) and to the toxic effects of TNFα (Takahashi et al., 1999), but does not improve resistance in lean mice.

Animal studies demonstrate that leptin is released during the early stages of an inflammatory response and that both TNFα and IL-1β are involved in the stimulation of leptin

expression and release. The transient nature of leptin stimulation by cytokines has been demonstrated in studies that treated cancer patients with repeated infusions of IL-1α (Janik et al., 1997) or TNFα (Zumbach et al., 1997).

Leptin does not appear to be essential for LPS-induced anorexia (Faggoni et al., 1997), therefore, the positive feedback loop between leptin and inflammatory cytokines may augment the early response to infection. As pro-inflammatory cytokines stimulate leptin during the early stages of the inflammatory response, and in vitro studies demonstrate that leptin activates macrophages, these experiments were carried out to determine whether leptin modified in vivo TNFα or IL-6 release in response to LPS endotoxemia in mice.

Materials and Methods

Experiment 1

Thirteen of each male and female db/db and +/+ C57BL/6J *Lep-r* mice, aged 10 weeks, were housed individually with free access to chow. On the day of experiment, starting at 8.30 a.m, the rectal temperature of each mouse was recorded and a small (100 ul) blood sample was collected by tailbleeding. Each animal received two intraperitoneal injections (i.p.): one was either 0.1 ml saline or 0.1 ml saline containing 10 ug LPS (Sigma Chemical Co.) and the other was either 0.1 ml PBS or 0.1 ml PBS containing 100 ug leptin (recombinant murine leptin, R&D Systems). Three of each gender and genotype were controls, given neither leptin nor LPS. The remaining groups each contained 5 mice. Exactly one hour after injection, a second, 50 ul, blood sample was collected by tailbleeding. Rectal temperatures were recorded 6 hours after injection and all LPS injected mice were decapitated. Trunk blood was collected, spleen, thymus, epididymal fat and retroperitoneal fat were weighed.

For each assay all samples were analyzed together in duplicate. Corticosterone was measured on serum from the baseline, 1 and 6 hour time points by RIA (Rat RIA kit: ICN Diagnostics Inc. Costa Mesa, CA). Free TNFα was measured in both baseline and 1-hour blood samples, diluted 1:25 in saline, by ELISA (Murine TNFα Quantikine kit; R&D Systems). Free IL-6 was measured in the 0 and 6 hour blood samples, diluted 1:25 in saline, by ELISA (Murine IL-6 Quantikine kit; R&D Systems). Leptin was measured on the 1 hour sample by RIA (Murine Leptin RIA kit, Linco Research Inc., MO) with serum from leptin injected mice diluted 1:100 in assay buffer and the serum from db/db control and saline injected mice diluted 1:20.

Experiment 2

Sixty-four 8 week old male C57BL/6J mice were purchased from Jackson Laboratories (MA). After 10 days of acclimation a baseline 100 ul blood sample was collected by tailbleeding, starting at 9.00 a.m. The next day the mice were divided into eight groups of 8 mice and, starting at 9.00 a.m., each mouse received two i.p. injections: one of either 0.1 ml PBS or 100 ug in 0.1 ml PBS, the other of 0, 2.5, 5.0 or 10.0 ug LPS dissolved in 0.1 ml saline. Exactly one hour later

a 100 ul blood sample was collected by tailbleeding and exactly 5 hours after injection the mice were decapitated. Trunk blood was collected, thymus, spleen, liver, epididymal fat and retroperitoneal fat pads were dissected and weighed. The epididymal fat was snap frozen for determination of leptin mRNA expression by Northern blot, as described previously (Harris et al., 1996). Corticosterone was measured on all samples, free TNFα was measured in both baseline and 1 hour samples, and free IL-6 was measured in baseline and 5 hour blood samples, diluted 1:25 in saline. Leptin was measured on the 5-hour sample.

Statistical Analysis

Statistically significant differences between treatment groups were determined by repeated measures analysis of variance or by two-way analysis of variance and post-hoc determination of least squares means at $P<0.05$ (SAS).

Results

Experiment 1

All of the measures made in this experiment were initially analyzed using gender as an independent variable, however, as there was no significant effect of gender, male and female mice were combined and the analysis was completed with genotype and leptin as independent variables. Rectal temperature, corticosterone, TNFα and IL-6 are shown in Figure 14 (see Appendix). The db/db mice showed a blunted response to the endotoxic stress. There were no significant effects of LPS or leptin on organ weights of either lean or db/db mice (data not shown).

Experiment 2

The effects of leptin and LPS on serum corticosterone are shown in Figure 15 (see Appendix) and on TNFα and IL-6 in Figure 16 (see Appendix). Corticosterone was significantly increased 5 hours after LPS injection, but not at 1 hour. At 5 hour leptin also had a significant effect (Leptin $P<0.03$, LPS $P<0.0001$, Interaction NS), suppressing corticosterone release in response to LPS. TNFα was essentially undetectable in baseline samples but LPS caused a substantial release of TNFα. When 1 hour values were compared by two-way analysis of variance, there was a significant effect of LPS on circulating TNFα concentration ($P<0.0001$), but no effect of leptin and no differences between the increasing doses of LPS. Although it appeared that leptin inhibited TNFα release in mice injected with 2.5 ug LPS, this difference was not statistically significant ($P<0.09$). Low levels of IL-6 were detectable in baseline samples and there was a significant effect of LPS on IL-6 concentration ($P<0.0001$), measured 5 hours after injection, but no effect of leptin and no interaction between LPS and leptin. LPS injection stimulated IL-6 concentrations in a dose-dependant manner with a significantly greater response to 10 ug LPS than to either 2.5 or 5.0 ug injections.

Organ weights and values for serum leptin concentrations and leptin mRNA expression, measured 5 hours after injection, are shown in Table 5. There were no significant effects of either LPS or leptin, or of an interaction between the two treatments, on liver, thymus or fat depot weights. Spleen weight was significantly increased by LPS treatment (Leptin NS, LPS $P<0.0001$, Interaction NS) but there was no dose effect. There were no differences in epididymal fat leptin expression. Leptin injection caused a significant elevation of serum leptin concentrations, which were still apparent 5 hours after injection, however, there was no effect of LPS on serum leptin concentrations in either the saline or leptin-treated animals.

Discussion

Although others have reported that leptin inhibits the corticosterone response to starvation stress (Ahima et al., 1996), we were unable to find any effect of leptin on the inflammatory response that resulted from septic stress. In addition, it has been reported that TNFα stimulates leptin expression and serum concentrations in animals that have been food deprived prior to the endotoxic challenge (Grunfeld et al., 1996). However, we were unable to detect any change in either leptin concentrations or adipose leptin mRNA expression in the LPS treated mice. In vitro studies have shown that leptin can augment an immune response by stimulating macrophages (Loffreda et al., 1998) but it appears that in vivo there is little effect of leptin on the inflammatory response measured under the conditions we employed and at the time points that we investigated.

The results from this study support the hypothesis that a fall in leptin signals energy deficiency (Flier, 1998). This drop in leptin initiates a series of responses that conserve energy. Results from this study suggest that an impairment of immune response may be typical of a starvation-induced response that can be reversed by leptin. In fed animals, where energy is readily available, leptin had no effect on the immune response to an endotoxic challenge.

The Effect of Endotoxic Stress on Adipose Tissue Metabolism in Rats

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Introduction

Endotoxic stress, such as that caused by administration of lipopolysaccharide (LPS), induces an inflammatory response, characterized by a transient stimulation of production of pro-inflammatory cytokines, such as TNFα and IL-6, by macrophages, fever, hypophagia and weight loss. In addition to an elevation of cytokines in the circulation there is stimulation of cytokine production by macrophages in tissue and these cytokines produce direct, localized effects on the cells. For example, genetically obese Zucker rats are hypersensitive to LPS with an increased rate of mortality compared with their lean controls due to liver failure caused by an abnormally high sensitivity to the TNFα produced by hepatic macrophages. The TNFα causes localized oxidative damage and liver failure (Uysal et al., 1997). TNFα is also produced by adipose tissue and it has been proposed that this cytokines acts directly on adipocytes to induce insulin

Table 5: Organ Weights And Leptin Levels In C57BL/6J Mice Treated With LPS And Leptin In Experiment 2

	0 ug LPS		**2.5 ug LPS**		**5.0 ug LPS**		**10 ug LPS**	
	Saline	*Leptin*	*Saline*	*Leptin*	*Saline*	*Leptin*	*Saline*	*Leptin*
Thymus (mg)	45 ± 2	49 ± 2	47 ± 3	46 ± 2	45 ± 2	46 ± 1	49 ± 2	47 ± 3
Spleen (mg)	71 ± 1^{A}	71 ± 2^{A}	82 ± 3^{B}	81 ± 3^{B}	83 ± 2^{B}	82 ± 2^{B}	83 ± 3^{B}	81 ± 3^{B}
Liver (mg)	375 ± 28	357 ± 31	358 ± 15	380 ± 35	397 ± 46	329 ± 15	392 ± 17	430 ± 56
Epididymal Fat (mg)	291 ± 12	313 ± 24	262 ± 21	298 ± 12	292 ± 2	290 ± 10	320 ± 19	285 ± 13
Retroperitoneal Fat (mg)	62 ± 4	55 ± 5	57 ± 5	66 ± 9	58 ± 5	54 ± 3	66 ± 5	58 ± 3
Serum Leptin (ng/ml)	1.9 ± 0.1^{A}	15.7 ± 3.6^{B}	2.5 ± 0.2^{A}	16.0 ± 2.8^{B}	2.1 ± 0.2^{A}	16.6 ± 4.2^{B}	2.2 ± 0.2^{A}	18.5 ± 6.0^{B}
Leptin Expression (Leptin mRNA:28S rRNA)	0.050 ± 0.014	0.035 ± 0.007	0.067 ± 0.018	0.044 ± 0.008	0.053 ± 0.018	0.067 ± 0.019	0.047 ± 0.006	0.042 ± 0.006

Data are means ± sem for groups of 8 mice. Values for a given parameter that do not share a common superscript are significantly different at $P<0.05$.

resistance and may be a factor contributing to the development of obesity induced diabetes (Yang et al., 1997).

The objective of this study was to determine whether stress-induced changes in adipose tissue metabolism were correlated with the production of pro-inflammatory cytokines TNFα and IL-6 in adipose tissue. Endotoxic stress induces a catabolic state in which both lean and fat tissue are lost. Some of the metabolic responses in the specific tissues may be secondary to promotion of localized cytokine production. In mice we had found that an intraperitoneal (i.p.) injection of LPS results in peak serum concentrations of TNFα one hour after injection and peak IL-6 six hours after injection. In this study we examined adipose tissue metabolism and cellularity at time points expected to correlate with peak circulating concentrations of these two cytokines.

Materials and Methods

Thirty-two male Sprague Dawley rats had free access to chow and water. On the day of the experiment food was removed from the cages at 8.00 a.m. and the rats were divided into 4 groups of 8 animals, matched for average body weight. One group received an i.p. injection of saline (0.1 ml/100 g body weight), the others received equivolume i.p. injections of 0.5 mg/Kg LPS given at times of 1, 2 or 6 hours prior to sacrifice. Injections were given at staggered time intervals so that all of the rats were killed between 1.00 and 3.00 p.m.

The rats were decapitated, blood was collected for measurement of TNFα, IL-6, leptin, insulin, glucose, free fatty acids, triglycerides and corticosterone. Epididymal fat was dissected and weighed. A small piece of tissue was fixed in osmium tetroxide for determination of cell size and number by Coulter Counter. A piece of tissue was snap frozen for extraction of total RNA, using Trizol reagent, for subsequent measurement of leptin mRNA expression by Northern blot. The remaining adipose tissue was minced and used to measure glucose oxidation and glucose incorporation into esterified fatty acids. Weighed aliquots of the minced fat were transferred to flasks containing 2 ml Krebs Ringer bicarbonate buffer, 10 mM HEPES, 2 mM glucose, 2% BSA, pH 7.45 and 0.3 uCi/umol U-^{14}C-glucose. The flasks were gassed with 95% O_2/5% CO_2 and sealed with rubber stoppers carrying center wells. They were incubated for 2 hours at 37°C and reactions were stopped by addition of 0.2 ml benzethonium hydroxide to the center well and 0.5 ml 1.0 N H_2SO_4 to the media. Thirty minutes later the center wells were transferred to scintillation vials for determination of oxidized glucose and the cells and media were extracted by Doles procedure for determination of glucose incorporation into esterified fatty acids. Results for glucose utilization are expressed per epididymal fat depot.

Results

The results for glucose oxidation by epididymal fat are shown in Figure 17 (see Appendix). There was no effect of LPS on glucose oxidation, which was significantly increased at both doses of insulin used. In contrast, there was significant inhibition of insulin-stimulated

glucose incorporation into fatty acids at both 1 and 2 hours after LPS injection, as shown in Figure 18 (see Appendix). This inhibition was associated with a reduction in the size of fat cells within the 80 – 100 um diameter range, as shown in Figure 19 (see Appendix). The inhibition of insulin-stimulated fatty acid synthesis was reversed by 6 hours after LPS injection and fat cell size distribution had also returned to control values at this time point. Results from the serum assays are shown in Figures 20 and 21 (see Appendix). LPS caused a significant stimulation of corticosterone at all time points after injection. Serum triglycerides were elevated 2 hours after LPS injection, a time that coincided with the inhibition of fatty acid synthesis. Unexpectedly, serum TNFα was at peak values at both 1 and 2 hours after LPS injection and IL-6 also peaked at 2 hours. This temporal pattern of cytokine response as different from that found in mice. Serum insulin, glucose, free fatty acids, and leptin were not different between groups. Epididymal fat leptin mRNA expression increased with time after LPS injection but the difference between groups did not reach statistical significance (data not shown).

Discussion

The results from this experiment indicate that there is a tendency for development of insulin resistance in adipose tissue of rats exposed to an endotoxic stress. The greatest effect was seen 2 hours after injection, when both TNFα and IL-6 concentrations were maximal. Further studies are needed to determine whether there is a direct relationship between circulating cytokine concentration and insulin resistance. The inhibition of insulin stimulated glucose utilization coincided with a reduction in fat cell size and an increase in serum triglyceride concentration, which may be representative of a lipolytic response in the tissue in addition to the inhibition of lipid synthesis. We found that the LPS caused a significant stimulation of the HPA axis, indicted by elevated serum corticosterone concentration, and it is likely that there was also activation of the sympathetic nervous system, which would promote lipolysis and reduction in fat cell size.

Additional studies are needed to clarify whether the insulin resistance and increased lipolysis are mediated by TNFα or by IL-6 and whether different fat pads respond differently to the stimulus. As it is likely that the sympathetic nervous system was stimulated by the endotoxin it is also possible that sympathetic innervation of the fat depots contributed to the response observed in these rats.

Stress-Induced Cytokine Signaling
Mary D. Boudreau

Introduction

Heterotrimer G-proteins mediate eukaryotic pathways that convert extracellular signals received by transmembrane serpentine receptors into changes in the concentrations of intracellular ions and small molecule second messengers. These signaling pathways control many aspects of neuroendocrine signaling. Upon activation, a receptor catalyzes the exchange of GDP for GTP in the alpha-subunit of a G protein (Gα subunit), and this exchange leads to

amplification of the initial signal. Essential to G protein signaling is the intrinsic temporal regulation of the cascade by the Gα subunit's ability to switch back to its inactive form through GTP hydrolysis. The regulator of G protein signaling (RGS) family of proteins plays a critical role in this process by enhancing the GTP hydrolysis rate of the Gα subunit (Watson et al., 1996). Although more than 20 RGS family members have been identified and many RGS proteins display distinct expression patterns, in general, most RGS proteins interact with several members of the G protein family and discriminate minimally among them. These findings suggest that the specificity of RGS proteins is likely to involve RGS domain interactions with proteins other than G-proteins. Support for this idea comes from experiments showing that cytokines might confer specificity of RGS action and use G-protein signaling to regulate the response of the nervous system to inflammation (Benzing et al., 1999). Interactions among the RGS proteins, the G proteins, and effector molecules may, therefore, determine whether a particular stress-induced signaling pathway elicits a restricted or relatively broad spectrum of physiological responses. At the beginning of this year, we began to examine the importance of RGS proteins in cytokine signaling and the importance of RGS proteins as determinants of biological functions in stress.

Materials and Methods

Sleep deprivation represents a physiological stressor that induces many of the symptoms of an acute phase inflammatory response, including weight loss, and altered thermal regulation (Youngblood et al., 1999). The first studies examined the effect of sleep-deprivation on systemic cytokine levels, brain fatty acid composition, and intracellular pathways that participate in and contribute to the production and release of TNF-α.

Male Sprague-Dawley rats, weighing 300-325g, were individually housed in Plexiglas cages with platforms of surface area 33 cm^2 for sleep deprived (SD) rats or with platforms of surface area of 320 cm^2 for tank control (TC) rats. Plexiglass shoebox cages were used for the cage control (CC) rat. The SD and TC cages were filled with 22° C water to within 2 cm of the top of the platform. Wire mesh lid covers on cages held food (Purina Rat Chow 5001; Purina Mills, Inc., St. Louis, MO) and a water bottle that were accessible to the rat placed on the platform. Morning body temperatures were measured on SD, TC and CC rats throughout the study. Following sleep deprivation for up to 96 hours, the rats were euthanized by carbon dioxide asphyxiation and blood was collected from the abdominal vena cava for cytokine analyses. In one experiment the lungs were immediately excised and lavaged with PBS and the macrophage-containing lavage fluid was stored at 4°C until subsequent enumeration and culture for cytokine analyses and the brain snap frozen in liquid nitrogen for subsequent fatty acids and protein analyses.

Macrophages were isolated, enumerated and monolayer cultures prepared from lung lavage fluid in RPMI cell culture medium (GIBCO BRL, Rockville, MD). Quadruplicate wells (1×10^6 magrophages/well) were cultured with or without lipopolysaccharide (LPS, 10mg/ml)

for 30 minutes at 37° C. Following the incubation period, spent culture medium was collected, centrifuged to remove cell debris and assayed for cytokine production. Total lipid fatty acids were extracted from rat brains by homogenization with the chloroform:methanol:butylated hydroxy anisole (BHT) solvent (2:1:0.02, v/v/v). Brain phospholids were fractionated from the total lipids by thin layer chromatography. Following saponification and methylation, fatty acid methyl esters were separated by gas chromatography. Identification of individual fatty acids was made by comparison of the retention time to that of known standards separated by gas chromatography under identical conditions.

Commercially kits (QuantikineM, R & D Systems Inc., Minneapolis, MN) were used to measure cytokines TNF-α and interleukin-6 (IL-6), in rat serum and macrophage culture supernatants. A commercially available antibody against long RGS proteins was used to establish a Western blot procedure for detecting the proteins, as shown in Figure 4 (see Appendix). And rats were injected peripherally with 0.5 mg/Kg LPS to determine whether changes in brain RGS protein could be detected in rats experiencing an inflammatory response, measured 6 hours after LPS injection.

Results

No differences in body temperatures, serum or macrophage cytokine concentration or brain fatty acid composition were observed in rat after 96 hours of sleep deprivation. In a study designed to determine whether cytokines were released as an early response to sleep deprivation, no differences in serum cytokine concentration was found in daily blood samples collected from rats that were sleep deprived for 104 hours. The failure to find a response to sleep deprivation was confirmed in Wistar rats, demonstrating that the previously measured hyperthermia in 96-hour sleep deprived rats was independent of the release of inflammatory cytokines. No differences in brain RGS protein concentration was detected in rats injected with LPS, compared with controls.

Discussion

The results from these studies failed to demonstrate a significant inflammatory response in rats exposed to extended periods of sleep deprivation and also failed to detect changes in RGS protein in rats that were experiencing an inflammatory response to the mitogen LPS. Therefore, this project has been abandoned in favor of more productive investigations.

Stress, Nutrition, and Immune Function

Mary D. Boudreau and Lisa M. Ballard

Introduction

Many stressful situations influence immune processes in humans and animals (Khansari et al., 1990). Common perceptions that chronic physical or psychological stress lowers resistance to infection and that infection develops more frequently during, or after, periods of stress suggest

that chronic physical or psychological stress is likely to impair the immune system. For example, experimental chronic stress prevents tumor rejection, increases tumor growth and reduces the severity of some autoimmune diseases (Visintainer, et al. 1982). However, the mechanisms by which stress impairs the activity of the immune system are not well known, and studies to determine the impact of stressful situations on various immunologic parameters often differ in their conclusions.

A significant decrease in the proliferative response to lymphocyte mitogens and impaired natural killer cell activity has been described in stressed animals. However, several chronic stressors increased adrenal weight and corticosterone secretion, but did not modify the splenocyte response to mitogens. Furthermore, an enhancement of immunological parameters after different types of stress has also been reported (Shurin, et al., 1994). These seemingly contradictory results can be explained by the fact that the response of the immune system to stress depends on several factors, such as the nature of the stress and the conditions and immune parameters analyzed.

The nutritional status of an individual can have an appreciable effect on response to infectious disease and other aspects of homeostasis under control of the immune system. Recommendations to reduce total fat and the level of saturated fats in the diet may have important effects on the immune response to both thymic-dependent and –independent antigens (Maki and Newberne, 1992). In general, in vitro studies suggest that diets containing polyunsaturated fatty acids (PUFA) may have immunosuppressive properties. However, dietary studies in which animals were fed PUFA-containing oils prior to *in vitro* assessment of T-lymphocyte functions have yielded contradictory results. One factor, which may explain previous contradictory results, is the type of serum used in cell culture. Fetal bovine serum (FBS) provides ingredients that support the maintenance and growth of cells *in vitro*, but it also contains uncharacterized factors, such as antibodies and growth factors, which may obscure assay results for specific responses.

The aim of these experiments was to analyze the effect of repeated restraint stress and high versus low fat dietary intake on cellular immune responses in adult male rats. A second objective was to compare effects of autologous serum and FBS on the ability of lymphocytes from stressed animals to respond to mitogens.

Materials and Methods

The purpose of these preliminary studies was to determine the optimal concentrations of mitogens and cells for use in mitogen-stimulated proliferation assays and to determine whether FBS and autologous rat serum exerted differing effects on the response of splenocytes to mitogens.

Spleens and blood were collected from male Sprague Dawley rats that had had free access to chow. The serum was separated by centrifugation for 20 min at 3000 rpm and 4^0C and was

sterilized by filtration through graded glass fiber and then though a 0.2μm filter cartridge. Serum was held at 4^0 C until added to cell growth medium. A single-cell splenocyte suspension was prepared by mincing the spleen followed by gentle mashing of the minced spleen pieces between the frosted ends of two glass slides. Cell debris was allowed to sediment and the splenocytes were depleted of erythrocytes by use of a red blood cell lysis buffer (Sigma) followed by two washes in PBS. Splenocytes were resuspended in 5 ml RPMI-1640 complete media which contained antibiotic/antimytotic (100 units/ml penicillin and streptomycin and 0.25 μg/ml fungizone), 2mM L-glutamine, 25mM HEPES, 2mM sodium pyruvate, 50mM β-mercaptoethanol , and 10% autologous serum or FBS. Spleen cell enumeration and viability were assessed by Trypan blue exclusion in a hemocytometer. Dilutions of splenocytes were made with complete RPMI-1640 to yield 2 x 10^6 cells/ml.

To measure lymphocyte proliferative response to mitogens, 100 μl of RPMI complete media containing 2 x 10^6 cells/ml/rat were distributed in triplicate wells of a 96-well culture plate and incubated for 72 hours with either100 μl of an optimum concentration of mitogen in complete media or complete media alone at 37^0 C in a humidified 5% CO_2 chamber. Following initial incubation period, 20 μl of 5 mg/ml of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) were added to each well. After culturing for another 4.5h, 100 ul of 10% SAS-0.01 N-HCl solution was added to each well and then the micro-culture plate was re-incubated overnight. A_{595} was measured using a BioRad plate reader.

Experiment 1: The Effects of Repeated Restraint Stress on the Responses of Rats Fed Low or High Fat Powdered Chow Diets

This study examined the proliferative response of spleen lymphocytes taken from rats that had been fed low or high fat diets. The objective was to confirm previous measures made by Dr Horohov that indicated an interaction between diet and stress, in which stress suppressed the proliferative response in cells from high-fat fed, but not low-fat fed rats.
Sixteen male Sprague Dawley rats had ad libitum access to rodent chow and water and were adapted to housing conditions for 10 days. Daily body weights were recorded for four days before the rats were divided into two weight-matched groups. One group continued on the powdered rodent chow, which provided approximately 10% of energy as fat. The other group of rats was fed a high fat diet consisting of 80% by weight powdered chow and 20% vegetable shortening, which provided approximately 40% of energy as fat. Daily food intakes and body weights were recorded for an additional two weeks and then the rats in each dietary treatment were subdivided into two weight-matched groups of four rats each. One group in each dietary treatment was exposed to repeated restraint, as described above, and the others were controls. At the end of restraint all animals were returned to their home cages. Rats were decapitated 24 h following the last restraint period. Spleen lymphocyte proliferation, using autologous serum, was determined, as described above.

Results

The results presented in Figure 22 (see Appendix) show that culturing lymphocytes isolated from rats fed rodent chow in medium containing FBS can partially mask the effects of mitogens on lymphocyte proliferation. In this study the response of lymphocytes to mitogen was greater when they were cultured in autologous serum than when they were cultured in FBS. These results were confirmed in a second study using a larger range of mitogen concentrations. In Experiment 1, there were statistically significant differences in food intakes of rats within a dietary treatment. Rats fed the low fat diet ate more food by weight, than those fed a high fat diet (data not shown). Rats fed the high fat diet gained more weight than low fat fed rats but the difference in body weight was not significantly different. When body weight change during the stress period was compared, there was a significant difference between animals fed the high fat diet and low fat restrained rats, as shown in Figure 23 (see Appendix). Although, in general, lymphocytes from spleens of restrained rats fed low fat diet tended to show a lower response to PHA and PWM stimulation, compared with controls, the differences were not significantly different. There were no differences in response of cells from high and low fat fed animals, (data not shown).

Discussion

The ex-vivo proliferation of lymphocytes has previously been used to demonstrate that high fat diets are immunosuppressive. Differences in protocols make it difficult to compare studies. One factor which may explain some of the contradictory results is the type of serum used in the assay. In Experiment 1 we used autologous serum at the same concentration level typically used for FBS (10%). It is possible that the serum concentration was not optimal for cell growth and proliferation. Further studies are needed to identify the optimal concentration of autologous serum and to confirm that FBS inhibits proliferation. We were unable to confirm the results from assays conducted by Dr. Horohov, but this may be due to differences in assay conditions, including the type of diet and serum used. A study is underway to compare the spleen lymphocyte response in rats that have been fed nutritionally balance high and low fat diets and autologous serum and FBS will be compared in the same experiment. Results from this study will provide more complete information regarding the effect of serum in addition to the interaction between diet and stress on immune function.

Another study is planned to examine the effect of different periods of restraint on immune functions in adult male rats. Rats will be fed a low fat diet (10% of energy) and restrained for two different schedules, either 2 h per day for 2 days or 6 h per day for 4 days. Plasma corticosterone will be measured as an index of stress, and lymphocytes will be cultured in medium containing autologous or FBS

C. Key Research Accomplishments

- Identification of naturally occurring urocortin antisense mRNA, which may regulate expression of sense urocortin mRNA and thus the response to stress.

- Demonstration of a substantial increase in β-receptor number in adipose tissue during the post-stress period in rats that have been exposed to repeated restraint stress. This change in receptor number may provide the biochemical basis for the shift in body composition that is observed in rats during the period following stress.

- Demonstration of an increased stress-responsiveness in mice that overexpress agouti protein. These observations implicate the melanocortin system in the regulation of behavioral and physiological responses to stress.

- Development of a probe for measuring CRF_2 receptor. This probe was used to demonstrate that the failure of rats to respond to chronic infusions of CRF into the brain was not due to a change in receptor expression but may be attributed to an up-regulation of the CRF binding protein.

D. Reportable Outcomes

Manuscripts

1. Youngblood, B.D., G.N. Smagin, P.D. Elkins, D.H. Ryan, R.B.S. Harris. 1999. Sleep deprivation and valine effect spatial learning and brain 5-HT metabolism. Physiol. Behav. 67: 643-649.
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6. Harris, R.B.S., D. Dunning, M. Boudreau, T. Mitchell and D.H. Ryan. In vivo effects of leptin on the inflammatory response to endotoxin in db/+ and db/db mice. Am. J. Physiol. (submitted).
7. Truett, G.E. J.A. Walker and R.B.S. Harris. A developmental switch affecting growth of fatty rats. Am. J. Physiol. (in press)

Chapters and Reviews

1. Smagin, G., R.B.S. Harris, and D.H. Ryan. The role of the locus coeruleus CRF-norepinephrine interaction in the behavioral response to stress. Pennington Nutrition Series 10: Countermeasures for battlefield stressors. LSU Press, Baton Rouge, LA. 2000
2. Harris, R.B.S., B. Youngblood, J. Zhou, G. Smagin, and D.H. Ryan. REM sleep deprived rats demonstrate a stress-induced impairment of spatial memory. Pennington Nutrition Series10: Countermeasures for battlefield stressors. LSU Press, Baton Rouge, LA. 2000
3. Harris, R.B.S., L.A. Howell, T. Mitchell, B.D. Youngblood, D.A. York and D.H. Ryan. Stress and macronutrient selection. In: Neural control of macronutrient selection. CRC Press, Boca Raton, FL 2000.
4. Harris R.B.S. Leptin – Much more than a satiety signal. Ann. Rev. Nutr. Vol 20. 2000

Abstracts

1. Zhou, Y., L.A. Howell, G.N. Smagin. D.H. Ryan and G.N. Smagin. Study of central regulation of stress responses in Apolipoprotein E-deficient mice. Neuroscience, 1999.
2. Mitchell, T.M., J. Simpson and R.B.S. Harris. Leptin does not prevent stress induced weight loss in rats. Neuroscience, 1999.
3. Youngblood, B.D., D.H. Ryan and R.B.S. Harris. The effects of sleep deprivation and sleep rebound on spatial learning and brain serotonin metabolism in rats. Neuroscience 1999.
4. Smagin, G.N., X. Yan, D.H. Ryan and R.B.S. Harris. Urocortin mRNA levels in the hypothalamus and midbrain are modulated by restraint stress and glucocorticoids. Neuroscience 1999.
5. Harris, R.B.S., B.D. Youngblood, D.H. Ryan. Prior food restriction does not prevent stress-induced weight loss in rats. Exp. Biol. 2000
6. Boudreau, M.D., D. Dunning, T.M. Mitchell, D.H. Ryan, R.B.S. Harris. In vivo effects of leptin on LPS inflammatory response. Exp. Biol. 2000.

Presentations

1. Ruth Harris served as an ad hoc reviewer for the NIH Endocrinology Study Section in February.
2. Ruth Harris was appointed to the editorial board of the Society for Experimental Biology and Medicine for 3 years from January 2000.
3. Ruth Harris presented a seminar "Stress and body weight regulation" at the University of Georgia in March 2000.

Degrees Obtained

1. Jun Zhou received her Ph.D. from the Department of Veterinary Physiology, Pharmacology and Toxicology, LSU School of Veterinary Medicine in August 1999. Her thesis work was supported by this grant and was entitled "Effects of repeated restraint stress on tissue metabolism in rats".

Employment Opportunities

1. Jun Zhou was a graduate student and is now employed as a post-doc by PBRC.
2. Gennady Smagin was an Instructor at PBRC and is now a Research Assistant Professor in the Dept. Pharmacology at LSU Health Sciences Center, Shreveport.
3. Patrick Awenowicz was a post-doctoral fellow at PBRC and is now a sales representative for the Life Sciences Division of Sigma Chemical Company.

E. Conclusions

The results of studies described above provide new information on a number of aspects of the relationship between stress and behavior. The expression of a naturally occurring antisense mRNA transcript for urocortin may represent a mechanism by which stress-induced urocortin expression can be regulated in a site-specific manner. This would allow specificity of behavioral and physiological responses to stress. Demonstration of changes in β-adrenergic receptor number in adipose tissue 24 hours after the end of stress is one of the first observations describing a sustained physiological response during the post-stress period. It also helps to explain the changes in body composition that occur in the days immediately following the end of exposure to stress. We are currently investigating repeated restraint stress as a model for post-traumatic stress disorder, but, independent of that, it provides a unique model for investigating mechanisms that normally control body weight and body composition and how these regulatory systems are chronically disrupted by acute stress. Demonstration of the involvement of the melanocortin system in determination of the responsiveness to stress suggests that it may be used as a marker for stress responsiveness. Indications that animals in which the system is disrupted due to a genetic manipulation have an altered sensitivity to stress, whereas acute manipulation of the system does not change stress responsiveness, strengthens the argument for this system being used as a genetic marker for stress responsiveness.

E. References

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IV. Stress, Nutrition and Work Performance

A. Introduction

Our overall objective is to conduct clinical research, which evaluates the efficacy of nutritional approaches that could enhance physical performance under conditions of extreme physical stress and/or energy deficiency. The primary dependent variables for these studies are muscle endurance, strength and anaerobic capacity. In this annual report we outline a study completed in the last year which, tested the hypothesis that consumption of branched chain amino acids can delay exercise time to fatigue. The rationale for this study is described below.

The development of fatigue as a result of prolonged exercise has been researched extensively but the specific mechanisms of fatigue still remain uncovered. This is illustrated by the fact that a precise definition of fatigue is still debated. It is loosely defined as the inability to maintain an expected power output, or a loss of force output, in response to voluntary effort that leads to reduced performance of a given task. This definition of fatigue still shadows the complexities of the etiology of fatigue.

It is this complexity and lack of consensus as to the etiology of fatigue that has separated fatigue research into two schools of thought: peripheral and central. Peripheral fatigue mechanisms are defined as metabolic and electrophysiological processes that occur independent of neural drive, whereas central fatigue mechanisms are considered a disruption in the course of the voluntary signal transmission from the brain to the motor neurons.

Peripheral fatigue has received the bulk of scientific attention. Nonetheless, there is a growing consensus that fatigue during prolonged exercise involves both central and peripheral components. It appears that a symphony of systems work together to elicit fatigue and research is now more focused on determining to what degree each system contributes to fatigue.

Several biochemical mechanisms for the development of central fatigue have been suggested, including the accumulation of ammonia in the brain during exercise (Okamura, 1987), and changes in the brain monoamine concentrations (Acworth, 1986). Increased serotonin production (levels) in the brain have also been implicated in the development of central fatigue. Serotonin (5-HT) is a neurotransmitter that has been linked to many human behaviors including: depression, hunger, perception, sleep and fatigue (Bhatti, 1998; Voderholzer, 1998; Ho, 1998). In the rat, exercise time to exhaustion has been shown to be decreased in a dose-related fashion in response to administration of a serotonin agonist (Bailey, 1992). On the other hand, a serotonin antagonist increased exercise time to exhaustion in rats (Bailey, 1993). In humans, cycling time to fatigue was reported to be decreased with administration of paroxetine, a serotonin re-uptake inhibitor (Wilson & Maughan, 1992). Thus, these studies provide reasonable support for a central component to fatigue during prolonged exercise and warrant further investigation.

Exercise Induced Biochemical Alterations and Serotonergic Related Central Fatigue

The amino acid tryptophan is the direct precursor for serotonin synthesis (Carlsson, 1972). It is found in both the free and bound (to albumin) forms in blood. During prolonged exercise, plasma free fatty acid levels rise and displace tryptophan from albumin resulting in increased free tryptophan levels (Chaouloff, 1997). Free tryptophan is available for transport across the blood brain barrier and subsequent serotonin synthesis. Branched chain amino acids (BCAA) are known to compete with the carrier proteins for tryptophan transport across the blood brain barrier (Fernstrom, 1972), but their circulating levels decline during prolonged exercise. Thus, these two metabolic phenomena can result in an increased potential for tryptophan transport and serotonin synthesis and possibly contribute to the development of fatigue during prolonged exercise. While there is no direct data in humans proving that this scenario exists, it is known that tryptophan infusion shortens exercise time to exhaustion in horses (Farris, 1998).

Nutritional Modification of Serotonergic Induced Central Fatigue

The involvement of tryptophan and serotonin production in the development of fatigue during prolonged exercise is an attractive hypothesis because it opens up the possibility that such

a central component could be nutritionally modified. For example, glucose administration during exercise is known to blunt increases in free fatty acids, while BCAA ingestion could prevent a decline in plasma BCAA concentration. Indeed there is a significant amount of evidence for the former strategy to prevent fatigue during prolonged exercise; however, the proposed mechanism has been increased substrate availability (Coggan, 1989; Coyle, 1983; Coyle, 1986) rather than prevention of central fatigue (Davis, 1992). Far fewer studies have attempted to delay fatigue with BCAA ingestion. To date, the results have been equivocal with some showing a delay in exercise fatigue (Mittleman, 1998; Blomstrand, 1991; Blomstrand, 1997) and others showing no effect (Van Hall, 1995; Blomstrand, 1995; Madsen, 1996).

Rationale

One limitation of earlier studies has been the timing of BCAA ingestion. Branched chain amino acids have been consumed either prior to or during the exercise bout as well as consumed shortly after the onset of exercise (van Hall, 1995; Blomstrand, 1996; Madsen, 1996). It seems that BCAA feeding would have the greatest potential for delaying fatigue if these amino acids were to be administered late in exercise when central components are making their greatest contribution to the overall development of fatigue. Furthermore, few studies have addressed whether glucose + BCAA ingestion is more effective than glucose alone at delaying fatigue and no study has controlled for a general amino acid effect by administering essential amino acids which have no known ergogenic effect. Therefore, the purpose of this study was to determine whether ingestion of a glucose + BCAA drink at fatigue prolongs additional exercise to a greater extent than glucose ingestion alone or a glucose drink containing the essential amino acids histidine, threonine and methionine. Well trained cyclists were tested because they routinely cycle to the point of, or even beyond fatigue making them the ideal candidates to test the hypothesis that ingestion of a glucose + BCAA drink is more effective than glucose alone at delaying fatigue during prolonged exercise.

B. Body

Methods

Subjects. A total of 8 male subjects participated in the study (Table 1 – see Appendix). All were highly motivated, well-trained endurance cyclists who frequently cycled to fatigue over prolonged periods (i.e., 2 - 4 hrs). Subjects were involved in a regular training regimen that included at least three training rides per week. Each volunteer was also required to complete a clinical screening, which included: medical history, electrocardiogram, physical examination, urinalysis and standard blood chemistries. Prior to participating the subjects were informed of the purpose and the potential risks of the study and written consent was obtained. This study was approved by the PBRC Institutional Review Board.

Protocol Design. Prior to any experimental testing, subjects completed a maximal oxygen consumption procedure (VO_2 max) to ensure that all subjects had a VO_2 max of at least 50 ml/kg/min. Body composition was determined by dual energy x-ray absorptiometry (DEXA).

Exercise on the day before an experimental trial was limited to 40 minutes of stationary cycling in the laboratory at 70% of VO_2 max. Three days prior to an experimental trial, all meals were provided to ensure adequate calorie and carbohydrate intake (i.e., CHO intake of 6g/kg body weight).

Each volunteer completed four experimental trials. The first trial was used to familiarize subjects with the testing procedure, and to ensure that the subjects could cycle at 70% of VO_2 max for at least 60 minutes. This first trial was conducted as described below except that blood samples were not drawn.

On the day of testing, subjects reported to the testing center following an overnight fast. On arrival subjects completed a mood state questionnaire (PANAS; Watson & Clark 1988). Next, a flexible Teflon catheter was then placed in an antecubital vein for blood sampling, and was maintained clear with a slow drip of sterile saline. Following catheterization, a baseline blood sample was obtained while seated. Subjects warmed up for 5 minutes at 100 watts, and then cycled to fatigue on an electronically braked stationary cycle ergometer (Lode; Groningen, The Netherlands) at a workload that corresponded to 70% of VO_2 max (*Bout 1*). Fatigue was defined as the inability to maintain a pedaling rate of greater than 50 rpm at the set workload. One individual was responsible for the motivation of subjects during all testing. Subjects were cooled with a fan and allowed to drink water at will. Heart rate from a heart rate monitor (Vantage XL, Polar Electronics) was recorded every 10 minutes, and breath by breath oxygen (VO_2) consumption and carbon dioxide production (VCO_2) was measured for 3 minutes every 30 minutes using a Sensormedic Metabolic Cart. From that measurement respiratory exchange ratio was calculated . Rating of perceived exertion (RPE) was determined every 30 minutes. Venous blood samples (15ml) were obtained after the RPE at 30-minute intervals and at fatigue.

After fatigue was reached (failure to maintain >50 rpm), a 20 minute rest period began. Experimental treatment was applied within the first 5 minutes of the rest period. The treatments were: (1) 400 ml of a carbohydrate drink, (2) BCAA (leucine, isoleucine, valine) added to the carbohydrate drink, or (3) three essential amino acids (threonine, histidine, methionine) added to the carbohydrate drink. These treatments were randomized and administered in a double blind fashion. Following the rest period the subjects performed additional exercise at the same intensity (70% VO_2 max) until they fatigued a second time (*Bout 2*). Heart rate, expired air (for VO_2 and RER determination) and venous blood samples were collected at 5, 10, and 15 minutes of exercise. At the time of the second fatigue (failure to maintain >50 rpm), a final blood sample was taken and time to fatigue was recorded.

The three experimental drinks were isovolumic (400ml) and isocaloric (344 kcal). The carbohydrate drink contained 86 grams of Powerade™ in 400 ml of water (CHO). The BCAA drink contained 75 grams of Powerade™, 5.2 grams leucine, 2.6 grams isoleucine, and 3.2 grams valine in 400ml of water. The essential amino acid (EAA) drink was used to discern the effects of BCAA vs a general effect of amino acids and contained 75 grams Powerade™, 4.0 grams threonine, 3.8 grams histidine, and 3.2 grams of methionine in 400 ml water. Powerade™ is a

commercially available "sports drink" and is made up primarily of glucose polymers and sucrose. USP amino acids were purchased from Seltzer Chemicals.

VO_2 max Determination. The test was conducted on an electronically braked cycle ergometer using an incremental work protocol. The bicycle ergometer workload was preset at 150 watts and the workload was increased by 50 watts every 2 minutes until 400 watts (12 minutes). At 400 watts the workload was increased by 25-watt increments every minute until exhaustion. VO_2 and VCO_2 were continuously monitored using a breath by breath gas analysis system (Sensor Medics Vmax29). Submaximal workload and VO_2 relationships determined during this test were used to predict a workload which corresponded to 70% of each subjects' VO_2 max. For assurance that VO_2 max was attained, two of the following three criteria were met: 1) a plateau in VO_2 with increasing workload, 2) maximum heart rate within 10 beats of the age predicted maximum heart rate, and 3) RER greater than 1.10.

Body Composition Assessment. Whole body dual-energy X-ray absorptiometry (DEXA) was used to determine lean body mass (LBM) and percent body fat. This measurement was made on a Hologic model QDR-2000 DEXA scanner (Waltham, MA).

Analytical Methods. Blood samples were analyzed for serum glucose and free fatty acids on a Beckman Synchron CX5 automated Chemistry analyzer. Serum prolactin was measured with a two-site chemiluminescent immunometric assay (Diagnostic Products Corp., Los Angeles, CA) and used as a surrogate measure for brain serotonergic activity (Fischer, 1991).

Statistical Analysis. Data are presented as mean ± SE and statistical significance was set at $p < 0.05$. Data were analyzed using single or doubly repeated measures design analysis (SAS for Windows version 6.12, SAS Cary, North Carolina).

Results

Responses during Bout 1. Time to fatigue during bout 1 was quite reproducible and not different for the three trials averaging 91.7 ± 9.8 min. for CHO, 79.1 ± 3.0 min. for BCAA and 79.8 ± 7.6 min. for EAA. After 60 minutes of exercise VO_2 averaged 3.28 ± 0.16 l/min and was not different between trials. This represented an exercise intensity of 69.7 ± 1.3%, 68.0 ± 2.5%, and 71.0 ± 0.9% for CHO, BCAA and EAA, respectively. RER averaged 0.84 ± 0.10 at 60 minutes of exercise and was not different between trials. Perceived exertion was slightly but significantly lower ($p < 0.05$) after 60 minutes of exercise during the CHO trial (16.1 ± 0.7 vs. 17.0 ± 0.7 and 16.8 ± 0.8 for BCAA and EAA, respectively). Serum glucose, FFA, and prolactin levels are presented in Table 2 (see Appendix). From baseline to fatigue serum glucose was unchanged and FFA were increased in all three trials. For prolactin, there was a significant time x treatment interaction ($p < 0.05$) with serum levels increasing from baseline to fatigue in CHO and EAA but they remained unchanged during the BCAA trial.

Responses during Bout 2. After ingestion of an experimental drink and a 20-minute rest period, additional exercise on the cycle ergometer was performed until fatigue. There was no significant difference in exercise time to fatigue between trials with subjects going the longest during the BCAA trial followed by CHO and then EAA (Figure 1 – see Appendix). Interestingly, five out of the eight subjects had their best performance after receiving BCAA while three of eight performed best after CHO alone (Table 3 – see Appendix). Perceived exertion after 10 minutes of additional exercise was not different between trials and exercise intensity was maintained at 69.2 ± 1.8% of VO_2 max and was not different between trials. Serum glucose and FFA levels prior to bout 2 were higher than what was observed at fatigue during bout 1. However, after five minutes of additional exercise glucose and FFA levels began to decline and remained lower than bout 2 baseline levels throughout exercise (Figure 2 – see Appendix). Serum prolactin levels were not different at baseline prior to bout 2; however, compared to the BCCA trial prolactin levels were higher during exercise after ingestion of CHO and EAA (Figure 2 – see Appendix; time x treatment interaction, $p < 0.01$).

C. Key Research Accomplishments

- Addition of branched chain amino acids to a carbohydrate drink does not interfere with the ability of a carbohydrate drink to enhance exercise performance. In fact, in some individuals ingestion of a carbohydrate drink containing branched chain amino acids may be more effective at delaying fatigue than carbohydrate alone.

- Branched chain amino acid ingestion seems to reduce prolactin secretion during exercise. This is suggestive of reduced serotonergic activity indicating that if central fatigue (emanating from serotonergic systems) plays a significant a role in the overall development of fatigue during prolonged exercise, then branched chain amino acid ingestion may be one way to temper this component.

D. Reportable Outcomes

Manuscripts

1. Zachwieja, J.J., D.M. Ezell, A.D. Cline, J.C. Ricketts, P.C. Vicknair, S.M. Schorle, and D.H. Ryan. Short-term dietary energy restriction reduces lean body mass but not performance in physically active men and women. *Revised and re-Submitted.*
2. Ricketts, J.C. and J.J. Zachwieja. Effects of creatine monohydrate supplementation on sprint swimming performance. *Submitted.*

Abstracts

1. Zachwieja JJ, Ezell DM, Cline AD, Ricketts JC, Vicknair PC, Schorle SM. An energy deficient diet reduces lean body mass but not performance in physically active men and women. *SEACSM Meeting.* Charlotte, NC., Jan. 27-29, 2000.

Presentations

1. J. J. Zachwieja. January 2000. "Carbohydrate, fat, and protein metabolism during prolonged exercise: integration of current concepts" Southeast Chapter American College of Sports Medicine Annual Meeting, Charlotte, NC.
2. J.J. Zachwieja. May 2000. "Interplay between exercise and nutrition for health and performance enhancement" John Stuart Research Laboratories, Barrington, IL.

Degrees supported by this award

1. Mr. Paul Moran, Masters Thesis. Primary Advisor, J.J. Zachwieja.

D. Conclusions

Results from the branched chain amino acid study, completed within the past budgetary year, suggest that ingestion of a carbohydrate drink containing branched chain amino acids may be as useful, and in some individuals more useful than carbohydrate ingestion alone at delaying fatigue during prolonged physical work. Further, it was shown that branched chain amino acid ingestion decreased prolactin levels during exercise. Prolactin secretion is a reasonable indicator of activation of serotoneric systems. Thus, if serotonergic activation of central fatigue plays a significant role in the development of overall fatigue during prolonged physical work, then branched chain amino acid ingestion may be one way to offset this component and in doing so improve physical performance. This study adds to a significant body of research evaluating the efficacy of branched chain amino ingestion for the purpose of improving exercise performance. Future studies should focus on between subject variability in prolactin response to prolonged exercise and determine if individuals with an exaggerated prolactin response respond more positively to branched chain amino acid ingestion.

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V. Nutrient Database Integration Laboratory

A. Introduction

Timely receipt of dietary data is critical in assessing soldiers' nutritional needs and how those may interrelate with other aspects of military performance. We currently have diversified our tasks to participate in planned field studies conducted by the Military Nutrition Division of USARIEM by providing assistance with and analysis of dietary intakes collected during military field studies. We advise in the planning of dietary collection protocols and subsequent analysis of collected dietary intake data. We can advise the principal investigators on dietary collection and methods to assure a quick turnaround of nutrition information needed for statistical analyses. We are working with USARIEM nutrition staff, data programmers, and other key personnel in the development of a shared database system for the collection and processing of dietary information. The objective is to generate finalized dietary data quickly, so that results can be disseminated in a timely fashion.

B. Body

Ray Allen continues to make progress on the MiDAS data entry system to facilitate more effective data management for field studies at USARIEM. Ray Allen and Catherine Champagne made several visits to USARIEM in August and September of 1999, to work on the set-up of the server system at USARIEM. This system will facilitate the entry of recipes and or food items (e.g. MREs) for use in field studies.

Ray Allen and Catherine Champagne visited USARIEM on December 6, 1999 for the purpose of demonstrating PBRC's Food Diary program. A working version of PBRC's MENu 98 program was installed on the USARIEM server on November 15, 1999 by Ray Allen. This allows the dietitians to enter recipes and menus as needed. Ray Allen and Catherine Champagne conducted a live demonstration of PBRC's Food Diary in order to solicit feedback in setting up the MiDAS data entry system. This eliminated the need for multiple databases allowing for a direct communication link to PBRC without compromising the need for appropriate networking security at USARIEM and will allow staff at USARIEM to access nutrient data from military studies using locally installed software (MiDAS). MiDAS will communicate with the nutrient database located at PBRC through the Internet. Testing of this methodology using Food Diary demonstrated that the methodology was feasible and data access times were acceptable. When completed, the final

version of MiDAS will be multi-purpose and accommodate many study needs without continual extensive modification for each field study.

PBRC participated in USARIEM's Navy shipboard study, Protocol H99-15 "Assessment of Energy Expenditure and Nutritional Status of Navy Women Aboard Ship" from February 19-March 3. Eric LeBlanc and Dawn Turner participated in the study for the entire length of time. Ray Allen, Catherine Champagne, and Jarrett Keller participated in set-up and close out of data entry. Processing of dietary intake data from the ship study is continuing. We project that the data will not be finalized until late summer. The food files need further work and completion of recipe input before any analyses can be run.

Catherine Champagne participated as part of a briefing of the Military on PBRC Military Nutrition tasks June 9, 2000. She discussed participation of PBRC in Military Field Studies investigating food and nutrient intakes of soldiers in garrison dining facilities and in field trials focused on needs during various types of training exercises.

PBRC plans to participate in a study entitled "A Comparative Study Between Special Forces Soldiers and Other Trained Army Units: Assessment of Dietary Intake and Energy Balance in Garrison and Evaluation of Nutritional and Other Risk Factors for Injuries and Illnesses." This protocol will be conducted at Ft. Carson, CO located in Colorado Springs. Ray Allen traveled to Natick to discuss the Ft. Carson Study with MAJ Maria Bovill on May 30, 2000. We are planning to again undertake coordination of data entry procedures and supply personnel for data entry and data collection. The Study is planned for July 4-17 and we will supply 3 recipe specialists and data entry personnel. This study will be somewhat similar to the Special Forces Study, which PBRC participated in at Savannah, GA during the summer of 1996. MAJ Bovill would like to dose Special Forces' soldiers and soldiers from a control group with doubly labeled water for the determination of energy expenditure. Additionally, she was also interested in visually estimating food consumption of 35 soldiers, again from each unit (total of 70), while consuming meals in their respective dining facilities. The visual estimation is planned for a total of seven weekdays, with diet records for other foods consumed outside of the dining facility as well as for recording weekend intake, when the facility is closed. We have been approached to participate by supplying recipe specialist personnel, in addition to our current data entry role, and we are planning to do so.

The manuscript submitted to Military Medicine has been accepted for publication. It is entitled "Incorporating New Recipes into the Armed Forces Recipe File: Determination of Acceptability." The scheduled publication date is February 2001.

C. Key Research Accomplishments

- Development of MiDAS data energy system: Ray Allen and Catherine Champagne made several visits to USARIEM in August, September, November and December of 1999 to work on the set-up of the server system at USARIEM

- PBRC participated in USARIEM's Navy shipboard study, Protocol H99-15 "Assessment of Energy Expenditure and Nutritional Status of Navy Women Aboard Ship" from February 19-March 3.

- PBRC plans to participate in a study entitled "A Comparative Study Between Special Forces Soldiers and Other Trained Army Units: Assessment of Dietary Intake and Energy Balance in Garrison and Evaluation of Nutritional and Other Risk Factors for Injuries and Illnesses. This protocol will be conducted at Ft. Carson, CO located in Colorado Springs

- The manuscript submitted to Military Medicine has been accepted for publication. It is entitled "Incorporating New Recipes into the Armed Forces Recipe File: Determination of Acceptability." The scheduled publication date is February 2001.

D. Reportable Outcomes

1. Karge, W.H., J.P. Deluca, L.J. Marchitelli, C. Champagne, R. Tulley, J. Rood, M.A. Paulos, and H.R. Lieberman. Pilot study on the effect of hyperimmune egg protein on elevated cholesterol levels and cardiovascular risk factors. Journal of Medicinal Food. 2(2): 51-63, 1999.
2. C.J. Baker-Fulco, W.J. Tharion, C.M. Champagne, and H.R. Allen. Dietary adequacy of two remote-site feeding systems. FASEB J, 14(4): A788, 2000.
3. Catherine M. Champagne, PhD, RD; Alice E. Hunt, PhD, RD; Alana D. Cline, LTC (Retired) U.S. Army, PhD, RD; Kelly Patrick; Donna H. Ryan, MD. Incorporating new recipes into the Armed Forces Recipe File: Determination of acceptability. Military Medicine, in press.

E. Conclusions

The issues of software development, licensing, and other related matters have been addressed and we are working to continually improve the MiDAS system for data entry during various field trials conducted with USARIEM-Military Nutrition Division. Data gathered from these field trials is presented annually at meetings such as Experimental Biology. One study was completed in February 2000 and another is planned for July 2000. One manuscript was published in the late fall on the Sargeants Major Study and the acceptability manuscript from PBRC's involvement in the Fort Polk Menu Modification Project will be published in early 2001.

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VI. Enhancing Military Diets

A. Introduction

One goal of the military is the promotion of healthy eating by soldiers (Committee on Military Nutrition Research, 1993; 1995). Based upon the recommendations of the Committee on Military Nutrition Research (1998), three nutrients were selected for study in this research project: antioxidant nutrients, folate, and calcium. The natural sources of these nutrients are: fruits, vegetables, and milk products, and it is desirable for humans to obtain these nutrients through natural sources, as opposed to dietary supplements (Institute of Medicine, 1999). This research project is designed to test methods for enhancing the consumption of fruits, vegetables, and milk products by soldiers. The first two studies will evaluate environmental, dietary, and psychological/behavioral factors that are associated with selecting fruits, vegetables, and milk products by soldiers enrolled in Basic Combat Training (BCT) at Ft. Jackson, SC. During the current year, the first study was completed, and the data have been analyzed. The research team is currently planning the design of the second study, which will test the efficacy of two different approaches for modifying the food selections of soldiers in BCT. The third study of this project will test strategies for modifying food selections of soldiers who are at a later stage of their military career.

The first study was an observational study of food selections and food intake of BCT soldiers during the period of January - March, 2000. In this study, there was no attempt to modify the eating habits of BCT soldiers. The primary aims of the study were: 1) evaluate changes in dietary habits across the eight weeks of BCT and 2) evaluate environmental, dietary, and psychological/behavioral factors that might be correlated with selecting fruits, vegetables, and milk products. The findings of this study will be used to guide the development of the interventions that will be tested in the second study. Secondary aims of the first study were: 1) evaluation of food intake and its relation to food selections, 2) evaluation of changes in body weight and the relationship of nutrition and body weight changes, and 3) assessment of the usage of current nutrition education by BCT soldiers. One objective of the study was to conduct the research so that it was minimally disruptive to the normal operations of BCT. Another objective was to conduct the study so that the identities of the research participants would remain completely anonymous. In order to accomplish these two objectives, the research team developed several innovative approaches for gathering the research data. This research project was approved by the Institutional Review Boards of the Army and PBRC.

B. Body

Participants

The soldiers of two companies from two different brigades (1/61 and 3/13) were studied. These two companies ate their meals at two different dining facilities (DFACs). During the first week of BCT, the soldiers were briefed about the study requirements by watching a film about the study and by asking questions that were answered by the Principal Investigator. A total of 139 soldiers volunteered to be in the study. This sample represented approximately 45% of the soldiers assigned to the two companies. When data were collected at the end of BCT, 92 soldiers (66% of the original sample) were available for study.

Experimental Design

As noted earlier, this study was designed to observe the behavior of BCT soldiers over the course of BCT without any type of intervention to modify the behavior of the soldiers. Two companies of soldiers, dining at two different DFACs, were observed for two days at the beginning of BCT and for two days at the end of BCT (eight weeks duration). Demographic information about the soldiers was collected, i.e., gender, ethnicity, and age, and differences in behavior were examined as a function of these demographic characteristics. Dependent variables included measures of: food selections, foods consumed, previous dietary habits, body weight and height, stress, anxiety, and depression. Environmental events that might influence eating behavior were also measured: time standing in line, time to select foods, time to eat, and total time in the DFAC. Another set of environmental variables was the placement of foods along the serving line.

Identification of Soldiers Using Barcode Technology

During this study we wanted to maintain the anonymity of the identity of each soldier, but the study design required that we correlate data collected during the first week of training with that collected during the eighth week. To accomplish these two objectives, we developed a novel method. This methodology used the barcode located on the back of the soldiers military ID as a means of tracking an individual soldier over time while maintaining the anonymity of the soldier. After scanning a number of these IDs, we found that the only identifying data that could be recognized from the scanned barcode was the soldiers rank. An example reading from a soldier's military ID is AD15FC8E1 Y74TQ70L.

It was determined that the barcode information was unique to each soldier and this enabled us to identify each soldier for the sake of correlating data without collecting the soldier's name or other information for later identification.

Collection of Timing Data Using Handheld Computers and Barcodes

One of the goals of this study was to determine the amount of time that soldiers spent in the dining facility. To accomplish this objective, we designed a technique using handheld computers equipped with barcode scanners, bar-coded clip-on badges and the soldier's military ID barcode.

Timing data were collected at four points in the DFAC. Location 1 was where the soldier first lined up to enter the DFAC. At this location, a member of our research team scanned their military ID and the soldier was issued a bar-coded clip-on badge that was also scanned. The soldier carried this assigned badge to Location 2 which was located near the beginning of the serving line. The bar code was then scanned by a second member of our team. Bar codes were scanned at two other locations: location 3, where the soldier had completed their food selection, and location 4, after the meal had been consumed.

The data collected at these four locations allowed for the determination of: 1) length of time the soldier spent in line before reaching the serving line, 2) time that the soldier spent in the serving line, 3) time spent eating, and 4) the total time spent in the DFAC.

Digital Photography of Food Intake

Another innovative technology developed for this study was the videotaping of food trays before and after food consumption with a digital video camera. This technique was developed to minimize the impact of our study on the soldiers' routine while still enabling the capture of their food selection and intake.

A digital video (DV) camera was set up at a centralized location between where the soldiers exited the serving line and the tray disposal station. While the soldiers were having their barcode badge scanned at location 3, their tray was placed on a table under the video camera. The bar-coded badge was clipped to the front of the tray for identification. The same procedure was followed when the soldier had finished eating to capture the amount of food that was uneaten. Each meal was captured on digital videotape and was labeled for later analysis by dietitians at PBRC.

The video captures were analyzed by connecting the DV camera to a computer using a video capture board. Images were then captured for each soldier's tray before and after consumption. Food selections were classified, and amounts of food taken and amounts of food consumed were estimated by three research associates. To accomplish this classification and estimation of quantity, a computer program specially developed for this study was developed. Foods were classified using the USDA food grouping system of the Food Guide Pyramid (The Food guide Pyramid, 1992). Twenty per cent of the photo captures were oversampled for all three research associates as a means of checking reliability/accuracy of food classifications and estimates of amounts of foods selected and consumed. Comparison of the foods classified by the three dietitians indicated that they agreed greater than 80% of the time. Soldiers generally

consumed about 90% of the foods that they selected. Correlations of the data pertaining to foods consumed indicated that the dietitians had a very high degree of agreement.

Analysis of the Digital Photographs of Foods Selected and Consumed

The food selection and consumption data for each soldier were compared to the recommendations for "active men" and "very active women" as defined in the USDA's Food Guide Pyramid (1992). The data indicate that most of the soldiers met the daily recommendations for meat, both at the beginning and end of BCT. Approximately three-fourths of the soldiers selected adequate vegetable servings, but only half consumed an adequate number of vegetable servings. At the beginning of BCT, approximately one-fourth of the soldiers consumed adequate servings from the grain and milk groups, while the number of soldiers who consumed adequate servings increased by the end of BCT. Few soldiers selected and consumed fruit both at the beginning and end of BCT.

When the data were examined for the soldiers as a group, they consumed nearly 10 ounces of meat per day, which is greater than the 7 ounces recommended by the USDA. Not surprisingly, men consumed more meat than did women. Average servings of grain were near the recommendations at the beginning of BCT and exceeded the USDA recommendations at the end of BCT. This increase in servings of grain was statistically significant. Additionally, men consumed more servings of grain than did women. At the beginning of BCT, the soldiers, on average, selected and consumed 2 servings of milk per day. The selection of milk products significantly increased by the end of BCT to meet the recommended 3 servings per day, on average. The soldiers in company 1/16 selected and consumed more servings of milk than did the soldiers in company 3/13. Blacks selected and consumed significantly less milk than other ethnic groups. For all soldiers, adequate servings of vegetables were selected and consumed at the beginning and end of BCT. Potatoes contributed to more than half of the vegetable servings at the beginning of BCT, and to half of the servings at the end of BCT. Most likely, this decrease in potato servings contributed to the slight, but significant drop in vegetable servings over the eight weeks of BCT. Men selected and consumed significantly more vegetables than did women. Fruit servings at the beginning and end of BCT were less than half of the 4 servings recommended. In fact, only 5% of the soldiers met the minimal standards for fruit servings at the beginning and at the end of BCT. At the end of BCT, females significantly increased the number of fruit servings selected and consumed, but still did not meet USDA recommendations, on average. Since the selection of fruits was so low, we investigated possible means of increasing fruit intake. One beverage that was popular among the soldiers were "fruit" drinks made from sucrose and fruit juices (20%). If this beverage were 100% natural fruit juices, and soldiers continued to select them at the same frequency, the impact would have been quite significant: per cent meeting USDA requirements would have increased from 5% to 30% for fruits selected and from 4% to 18% for fruits consumed.

Opinions of the BCT Soldiers

In order to assess the opinions and attitudes of the BCT soldiers about their dining experiences, a separate sample of soldiers were interviewed in small groups (6 to 10 soldiers per group) by two psychological researchers. In the first week of BCT, 42 soldiers were interviewed and in the eighth week of BCT a new sample of 35 were interviewed. The responses of the soldiers indicated that over 80% of the participants believed that healthy eating was either "pretty important" or "very important." In the DFACs, nutritional education information is posted throughout the facilities. This information includes posters of the Food Guide Pyramid and daily posting of the menu. At the beginning and end of BCT, an overwhelming majority of soldiers (> 85%), indicated that they did not use this information to guide their selections of food. Of all the various types of nutrition education they received, a large proportion of soldiers (55% in Week 1 and 37% in Week 8) reported that information provided by their Drill Sergeant was most useful. At the beginning of BCT, soldiers felt that the "time to eat" was the one aspect of dining that they would most prefer to change. By the end of BCT, the soldiers expressed significantly less concern about "time to eat" and were more concerned with the "variety of foods" that were served. From these interviews, we concluded that: a) soldiers in BCT are interested in healthy eating, and may be motivated to follow suggested dietary guidelines, b) BCT soldiers do not often use current nutrition education strategies when making food selections, c) interventions which emphasize the influence of the Drill Sergeants may be most effective, and d) BCT soldiers would like to have more information about the menus and they might use this information to make healthy food selections.

Environmental Factors Associated with Food Selections

One of the logistical goals of the DFACs is to efficiently move soldiers through the process of selecting and consuming foods. Ideally, the total time in the facility for each company should be no more than 30 minutes at each meal. As noted earlier, we timed movement (of individual soldiers) through the serving lines at each meal during two days during Week 1 and two days during Week 8 of BCT. Figures 1 and 2 (see Appendix) illustrate the findings of this time/flow analysis. Figure 1 (see Appendix) depicts the average total time spent in the DFAC, and the average time standing in line, in the serving line, and eating, during all three meals during week 1. Figure 2 (see Appendix) illustrates the same information during week 8 of BCT. In general, the movement of soldiers through the facility was similar in Weeks 1 and 8. Also, on average, soldiers were in and out of the dining facilities in less than 30 minutes. Breakfast required the least amount of time. Also, the total time in the dining facilities during lunch and dinner was greater in the 3/13 Starship facility, in comparison to the smaller 1/61 Rolling Pin facility. The primary determinant of this difference in time was that soldiers generally spent slightly more time standing in line (waiting to get to the serving line), in the Starship facility. The correlations of timing variables with food selections were generally moderate in magnitude. The most consistent finding was that greater time to select foods and less time standing in line was associated with more frequent selection of milk products. **From these observations, we concluded that modification of the time spent in the dining facilities was probably not a good target for intervention**. These data suggest while time to select foods and time to eat

foods, probably plays some role in determining food selections, it does not appear to be a potentially powerful factor.

Another environmental factor that was studied was the association of where a food was placed on the serving line and probability of selecting that food. Figures 3 and 4 (see Appendix) illustrate these associations for two meals. Figure 3 (see Appendix) illustrates these relationships at Breakfast during week 1 and Figure 4 (see Appendix) illustrates the same relationships during at dinner during week 1. For all meals, soldiers selected most of their foods during the first quarter of the serving line. This association was most evident for breakfast foods. It is important to note that the placement of foods was designed to have this effect. Entrees, vegetables, and breads were among the first items on the serving line, and not surprisingly, soldiers selected these basic staples. Examination of the data for dinner suggests that soldiers do select foods that are farther down the serving line with some frequency, e.g., beverages, desserts, and salads were fairly common selections toward the middle and end of the lines. These data suggest that placement of foods might be one factor that could be modified, though the team feels that this approach should probably be coupled with a nutrition information intervention that draws the soldier's attention to a "healthy food selection".

Body Weight Changes during BCT

On average, BCT soldiers lost about 4 lb. during the eight weeks of BCT. It is probable that soldiers lost fat mass and increased muscle mass, though this study did not study such changes in body composition. Figure 5 (see Appendix) summarizes the mean changes in body weight as a function of gender and ethnicity. In this sample, black females and "other males" (Pacific islanders, persons of Asian or Native American heritage), lost the most weight and white females lost the least amount of weight. On average, BCT soldiers were in the high range of "normal body weight".

Changes in Mood, Anxiety, and Stress

The soldiers in our sample completed several self-report measures at week 1 and 8 of BCT. Previous studies have found negative mood states such as depression, anxiety, and stress can be associated changes in healthy eating patterns or food habits. Therefore we chose to examine the correlations between stress, mood, and anxiety and food selections and food consumption.

Because stress represents the demands associated with coping with any type of change, several measures of stress were selected for use in this study. The Perceived stress scale (Cohen, Kamarck, & Mermelstein, 1983) was selected to measure global cognitive perceptions of stress (e.g. feeling overwhelmed). The Weekly Stress Inventory (Brantley, Jones, Boudreaux, & Catz, 1997) was selected to assess the number of stress events experienced and impact of commonly experienced stressful situations in normal daily life (e.g., had to wait in line, had argument with coworker). An attempt was made to find a measure of stress associated with military life but none was identified. In order to assess the unique experiences of the BCT soldier, the Military

Stress Scale was developed as a part of this study. It was believed that creating such an instrument would allow us to more accurately measure commonly experienced stressful situations associated with normal military life.

Two additional measures of psychological distress were added to measure emotional reactions other than stress. These were: the Beck Depression Inventory (short form) which measures both cognitive and physical symptoms commonly associated with depression and the Beck Anxiety Inventory (short form) which measures primary physiological arousal commonly associated with states of stress and anxiety (Beck, Epstein, Brown, & Steer, 1991). The scores and norms of each instrument are presented in Table 1.

Table 1: Average Scores on psychological measures at the beginning and end of BCT, relative to norms

	Week 1	**Norms**	**Week 2**
Weekly Stress Inv-Impact	118	25-175 (average)	99
Weekly Stress Inv-Events	39	10-50(average)	35
Perceived Stress Scale	26	24 (average)	24
Military Stress Scale	49		36
Beck Depression Inven	3	3 (minimal)	2
Beck Anxiety Inven	14	8-15 (mild)	11

On average, the soldiers were in the normal range at the beginning and end of BCT, on all measures except for the measure of anxiety, which stayed within a range of mild elevation. All measures showed decreased stress, depression, and anxiety over eight weeks of BCT, suggesting that there is a natural adaptation to the stress associated with BCT.

This effect was less pronounced in one company (1/61) because the baseline scores of anxiety and stress were lower for this company relative to the other company. Women were found to be significantly more anxious than men at the beginning and end of BCT. Tests of the association of these psychological variables with food selections indicated no significant correlations. Therefore, we concluded that modification of psychological factors was not a good target for modification of food selections.

Development of the Military Stress Scale

As noted earlier, we could not identify a measure of stress that was specific to BCT or the military environment. As a part of this study, we developed the Military Stress Scale (MSS) a 28-item questionnaire designed to assess minor stressful events during a one-week period. The MSS measures both the frequency and magnitude of daily stressful events. Respondents indicate all events that have occurred in the past week and rate the severity of stress experienced for each event. The perceived stress of weekly events is rated on a five-point Likert scale ranging from "1" (occurred but was not stressful) to "5" (very stressful). The MSS yields 2 scores: (a) Event scores, and (b) Impact scores. The Event score is the number of items rated as having occurred during the day. Event scores provide an objective measure of the frequency of stressful events

experienced, with high scores indicating the respondent experienced many common stressful events. The Impact score is the sum of the perceived stress rating values assigned to the items. Impact scores reflect the number of stressful events and one's personal appraisal of stressful events. High Impact scores indicate higher levels of perceived daily stress. The MSS requires an estimated 8th grade reading level, consistent with other military reading requirements.

Statistical analyses of these data found that the MSS was more strongly associated with other measures of stress than it was with other measures of negative affect (e.g. depression). Factor analysis indicated that six aspects of military stress are measured by the MSS:

1. Personal Evaluation (5 questions concerning stress about potential negative evaluation)
2. Personal Restriction (6 questions concerning the restrictions of "freedom")
3. Public Reprimand (5 questions pertaining to being reprimanded by officers, etc.)
4. Regimentation (5 questions about stress associated with leading a regimented life)
5. Knowledge Deficits (4 questions pertaining to failing military tests, studying, etc)
6. Physical Complaints (3 concerning physical discomforts common to BCT)

Tests of the stability of scores on the MSS over the eight weeks of BCT found that soldiers that were most highly stressed at the beginning of BCT were also the most highly stressed at the end of BCT. In conclusion, the results of this study suggest that the MSS has the statistical properties that are necessary to allow its use as a measure of stress associated with military service in BCT.

C. Key Research Accomplishments

- Digital photography methodology was developed as a means of measuring food selections and food intake in military dining facilities
- Use of palm held bar code readers was used to unobtrusively track data collection for individual soldiers across eight weeks of BCT
- Soldiers expressed an interest in learning about healthy nutrition, but did not report frequent use of current nutrition education used in BCT
- The selection of fruits by BCT soldiers was below minimal nutritional standards for very active adults
- Fruit selections and fruit intake might be enhanced by replacing "fruit" beverages with 100% fruit drinks
- Milk selections and intake improved significantly over the eight weeks of BCT, changing from below USDA recommended values at week 1 to above recommended values at week 8, on average

- Soldiers consumed adequate amounts of vegetables, on average, but a substantial portion of vegetable intake could be attributed to eating potatoes
- Healthy food selections tended to improve over the course of BCT
- Environmental factors such as time to select foods and placement of foods along the serving line had only modest associations with food selections and food intake
- Soldiers lost an average of about 4 lb. over the eight weeks of BCT
- A new measure of stress during BCT, the Military Stress Scale, was developed and found to be reliable and valid
- Stress, anxiety, and mood improved over the eight weeks of BCT
- Psychological status of the subject, i.e., stress levels, mood, and anxiety, were not significantly associated with food selections or food intake

D. Reportable Outcomes

A video teleconference is scheduled for July 25, 2000 to report the results of the study to key military personnel. During this video teleconference, the design and aims of the second study will be planned. A paper describing the digital photography methodology for measuring food selections and food intake has been submitted for presentation at the annual meeting of the North American Association for the Study of Obesity in October 2000.

E. Conclusions

The first of three studies has been completed and the data have been analyzed. The study found that BCT soldiers are interested in learning more about healthy nutrition, but are often not utilizing current nutrition education programs and information. They are selecting diets that are low in fruits and milk products at the beginning of BCT. The consumption of milk products improves during BCT, but fruit intake remains low. Environmental and psychological factors do not appear to be especially good targets for modification of the diets of these soldiers. The most promising means for modifying their diets appears to be: a) provision of more information and education about selecting fruits, vegetables, and dairy products, b) working with Drill Sergeants to use their influence to reinforce the selection of diets with more fruits, vegetables, and dairy products, and c) provision of 100% natural juices as a means of enhancing fruit intake.

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VII. Stress, Nutrition and Immune Function Laboratory

A. Introduction

Participation in physically rigorous military training results in both physical and mental stress (1). Some of the stressors associated with training include caloric deficiencies, sleep deprivation and exercise. Concomitant with these stresses is an alteration of a number of parameters of lymphocyte-related immunity (2-4). Though the significance of these *in vitro* alterations in immune responses is unclear (4), an increase in documented infection rates coincided with indications of compromised immune function in US Army Rangers course participants (5). Thus the characterization of the mechanism of stress-induced alterations in immune responses could lead to the development of preventative strategies for reducing this immunomodulatory effect and improving the health of the soldiers. Nutritional status can be a very important component of the stress response (6, 7, 8, 9). While moderate dietary energy restriction prolongs life span and enhances immune responsiveness (10), malnutrition is

associated with decreased antibody response to vaccines (7,11). On the other hand, excessive intake of nutrients also impairs immunity (12). This has led to the suggestion that the amount and type of dietary fatty acids can influence *in vitro* measures of immune function (13) and that dietary composition may need to be altered during stressful conditions (14).

In order to determine the effect of diet on stress-induced immune modulation we have utilized a rodent sleep-deprivation model to mimics some of the changes observed in the Army Rangers course (3). Prior work in our laboratory has demonstrated that splenocytes from rats subjected to 48-72 hrs of sleep deprivation exhibited profound alterations in their ability to respond *in vitro* to various mitogens. Interestingly, diets containing high levels of polyunsaturated fats could overcome this immunosuppressive effect. We now have examined the effect of sleep-deprivation and dietary fat on the ability of rats to respond to *in vivo* immunizations.

B. Body

Materials and Methods

Animals. Male Sprague-Dawley rats (600 g) were used in all experiments. Rats were caged individually with *ad libitum* access to food and water.

Sleep Deprivation. Rats were sleep deprived by placing them into pedestal-style cages in which a small platform is surrounded by water (15). Rats are allowed free access to food and water while on the platform but they cannot lie down.

Diet. Rats were fed either a low fat diet (LF), a safflower oil diet (SO) high in n-6 polyunsaturated fatty acids (PUFA), an olive oil diet (OO) high in monosaturated fatty acids, a coconut oil diet (CO) high in saturated fatty acids, or a menhaden oil (MO) diet high in n-3 PUFA. All diets were commercially prepared (Research Diets, Inc, New Brunswick, NJ) and contained all essential amino acids, vitamins, and 4% corn oil. Other oils were added to represent 30% of the calories in each of the test diets. The low fat dies (LF) had the 4% corn oil as its only source of fat. All diets were isocaloric with starch substituting for the fat in the low fat diet. Rats were fed pelleted diets except while in the sleep deprivation tanks where treated and cage control rats were fed liquid formulations of the specific diets.

Experimental Design and Analysis. Rats were placed onto the test diet two weeks prior to sleep deprivation and remained on the diet throughout the stress period. Control rats were housed in the same rooms but were not sleep deprived. Vaccinated rats received a single intramuscular injection of 100 μg of keyhole limpet hemocyanin (KLH) with alum adjuvant. Rats were injected immediately prior to sleep-deprivation. Control (non sleep-deprived) rats were injected with the same vaccine preparation at the same time as the stressed rats. Four rats were included in each group. The rats were sleep-deprived for 48-72 hrs and then returned to their cages. Necropsies were performed 1-month post vaccination and splenocyte cultures were

set up in quadruplicates. Sera samples were collected at necropsy. Each experiment was performed thrice. Results represent the average counts per minute (CPM) for the individual lymphocyte cultures for each of the four rats. Results were analyzed using a one-way analysis of variance with post hoc analysis using Student Neuman Keuls' multiple comparison test.

ELISA. Antibodies to KLH were determined by ELISA. Plates were coated with KLH (5 μg/well) and blocked with 1% fish gelatin. Serum samples were serially diluted and added to the plate in duplicates. Rabbit anti-rat-Ig conjugated with horseradish peroxidase was used to detect the rat antibodies. Plates were developed with the TMB substrate (KPL Laboratories) and read on a microplate reader.

Lymphocyte Proliferation. Splenic lymphocytes (2×10^5) were incubated for three days with mitogens (pokeweed (PWM), phytohemagglutinin (PHA) and concanavalin A (ConA) or five days with KLH (0.5 μg) at 37° C in a CO_2 incubator. Plates were pulsed for 4 hours with 0.5 μCi of ^{3}H-thymidine and the DNA harvested onto filter pads for liquid scintillation counting. Stimulation indices (SI) were calculated as CPM of stimulated cultures/ CPM of medium controls. All determinations were performed in triplicate.

Results

In our initial study the rats were fed a standard lab chow diet throughout the experimental period. This diet actually represents a low-fat diet as only 4% of the calories are derived from fats. As previously reported (15, 16), sleep deprivation resulted in a significant decrease in the weight gain both immediately following the sleep deprivation period (Figure 1 in the Appendix) and persisting for up to one month later (Figure 2 in the Appendix). To determine the effect of dietary fat on sleep deprivation associated weight loss, rats were placed on different diets for two weeks prior to sleep deprivation. The OO and MO diets resulted in a significant ($p<0.05$) increase in weight compared to the other diets (Figure 3 in the Appendix). While the other fat diets were not significantly different from the LF diet, there was a general trend for them to be higher. Despite these diet-related differences in initial weights, subsequent sleep deprivation resulted in similar weight losses amongst the different groups with only the MO group being significantly different from the others (Figure 4 in the Appendix; $p<0.05$).

To determine the effect of diet on the sleep deprivation-induced suppression of the lymphoproliferative response to mitogens, rats were pre-fed the diets for two weeks and either sleep deprived for 48 hours (SD) or kept in their cages (CC). There was a significant ($p < 0.05$) dietary effect on the lymphoproliferative response of the CC rats fed the MO diet (Figure 5 in the Appendix). Sleep deprivation of the rats on the LF resulted in a characteristic suppression of their response to the mitogen. By contrast, the mitogen responses of the rats on the other high diets fat diets were not affected by the sleep deprivation.

We also observed a decreased antibody response to a vaccine antigen in sleep deprived rats with the most profound effect in the 72 hour sleep-deprived group (Figure 6 in the

Appendix). The sleep-deprived rats also experienced a significant reduction in their antigen-specific lymphoproliferative response to the KLH (Figure 7 in the Appendix). To determine the effect of diet on this *in vivo* response, rats were pre-fed the different diets for 2-weeks, vaccinated with KLH and either sleep deprived for 48 hours (SD) or kept in their cages (CC). The rats remained on the diet for an additional month and their cellular and humoral immune response to the KLH then measured. Similar to the *in vitro* assays, the higher fat diets ameliorated the sleep deprivation-induced modulation of the humoral response to the vaccine (Figure 8 in the Appendix).

C. Key Research Accomplishments

- These studies demonstrated that sleep deprivation causes a profound alteration of both *in vitro* and *in vivo* immune responses. The altered *in vivo* response included both cellular and humoral immune responses to an alum-adjuvanted vaccine.

- These studies also provide the first evidence that the immunomodulatory effect of sleep deprivation can be offset by increasing the fat content of the diet.

D. Reportable Outcomes

A manuscript on the *in vitro* analyses has been submitted for publication and an additional manuscript on the *in vivo* effects is in preparation.

E. Conclusions

Participation in physically rigorous military training results in alteration of a number of parameters of lymphocyte-related immunity (2-4) that may result in an increased incidence of disease (5). While the mechanism of this immune modulation remains unclear, it is generally supposed that the multiple stressors associated with this training may be responsible for these effects (2-5). There have been numerous reports indicating that physical, chemical or psychosocial stress can result in immune modulation and an increased susceptibility to disease (for review see (17)). The adverse effect of stress on health and well-being is widely recognized (18). These and other studies have led to the concept that there are interactions between the immune system and the central nervous system and that the immune system may be modified by stress to produce inappropriate changes in immunocompetence (19). The demonstration of soluble mediators and receptors common to these two systems further supports this notion (20). Little information is available, however, regarding the specific interactions that may be involved in stress-induced immune modulation. The reasons for this uncertainty include the complexity of the stress and immune responses (21).

A number of model systems have been reported for studying stress-induced changes in immune function (22). Results from these studies have demonstrated that few generalizations are possible because the direction and/or magnitude of the effects of stress in modulating immune

responses clearly depends on the stressor, the nature of the immune response and the immune compartment in which it is measured, the time of sampling, a variety of host factors (e.g. species, strain, age, sex), and interactions among these several variables (21).

The possible contribution of sleep deprivation in the immune modulation associated with physically rigorous military training has been suggested (2-4). The adverse effect of sleep deprivation on immune function is likewise widely recognized (23). Studies in both humans and animal models have demonstrated multiple effects of sleep deprivation on immune function (24, 25). In our hands, sleep deprivation of the rat results in profound effects on both *in vitro* and *in vivo* immune responses. We previously reported a time-dependent effect of sleep deprivation on the ability of rat splenocytes to proliferate in response to the mitogens PHA, ConA and PWM, and that the addition of IL-2 to these cultures failed to overcome this inhibition, suggesting that sleep deprivation inhibits lymphocyte proliferation at a step following IL-2 binding to its receptor (26). Here we report that feeding the rats a high fat diet resulted in a modulation of the sleep deprivation effects depending upon the nature of the fatty acids in the diet. Thus diets high in polyunsaturated fatty acids (PUFA) overcame the reduced proliferative response of the sleep-deprived rats.

We have extended our *in vitro* work to include an *in vivo* analysis of the effect of sleep deprivation and dietary fat on the ability of the rat to respond to a novel antigen vaccination protocol. As seen in earlier studies, sleep deprivation resulted in both a persistent weight loss (15) and a diminished response to vaccination, affecting both the humoral and cellular responses to the vaccine. These results indicate that our previous *in vitro* observations do coincide with a diminished capacity to respond to antigen *in vivo*. Similar to our *in vitro* studies, increasing the fat content of the diets could overcome the immunosuppressive effect of the sleep deprivation. Interestingly the higher fat diets failed to prevent the weight loss associated with the sleep deprivation period. Together these results indicate that the sleep deprivation-induced weight loss is not directly correlated with the alteration in immune function.

Our results are similar to reports of dietary supplementation with PUFA preventing immunosuppression after surgical (27,28) and burn trauma (29). Given the immunosuppressive nature of these diets, such a protective effect seems paradoxical. Nevertheless a similar effects of dietary fat on exercise-induced immune modulation has also been reported (30). Though these findings remain controversial (31,32), no systematic analysis of dietary fatty acids on stress-induced changes in immune function has been reported.

The mechanism of dietary fat effects on immune function remains uncertain. While eicosanoid pathways can be altered by increasing n-3 PUFA, it is also clear that these fatty acids can also elicit their effects by eicosanoid-independent mechanisms (33). Alterations in CD4:CD8 ratios have been associated with feeding diets high in n-3 PUFA (34). While we did not examine lymphocyte subset distributions in the current studies. Likewise possible effects of the diet on macrophage function were not assessed, though others have reported significant alterations (35).

Perhaps the most interesting observation made from these studies is the high degree of susceptibility to stress-induced immune modulation associated with the low fat diet. Give that most rodent diets are low fat (36) compared to human diets, the possible consequences of utilizing such diets should be considered in future experiments.

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VIII. Metabolic Unit Project

During this year of the grant (7/1/99-6/30/00) this project was inactive.

APPENDIX

TASK I: CLINICAL LABORATORY FOR HUMAN AND FOOD SAMPLES

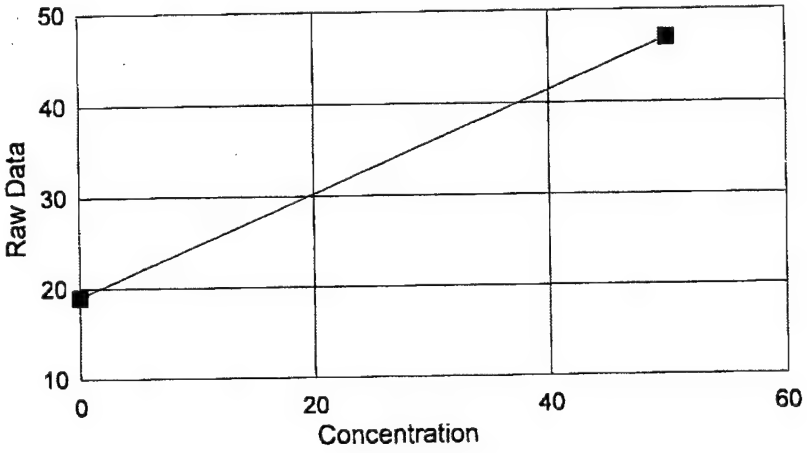
Minimum Detection Limit Calculation
Instrument: Immulite
Test: IGF-2

0 (A) Calibrator	rate found
1	18
2	19
3	19
4	20
5	
6	
7	
8	
9	
10	
Mean	19
SD	0.8164966
Mean + 2SD	20.632993

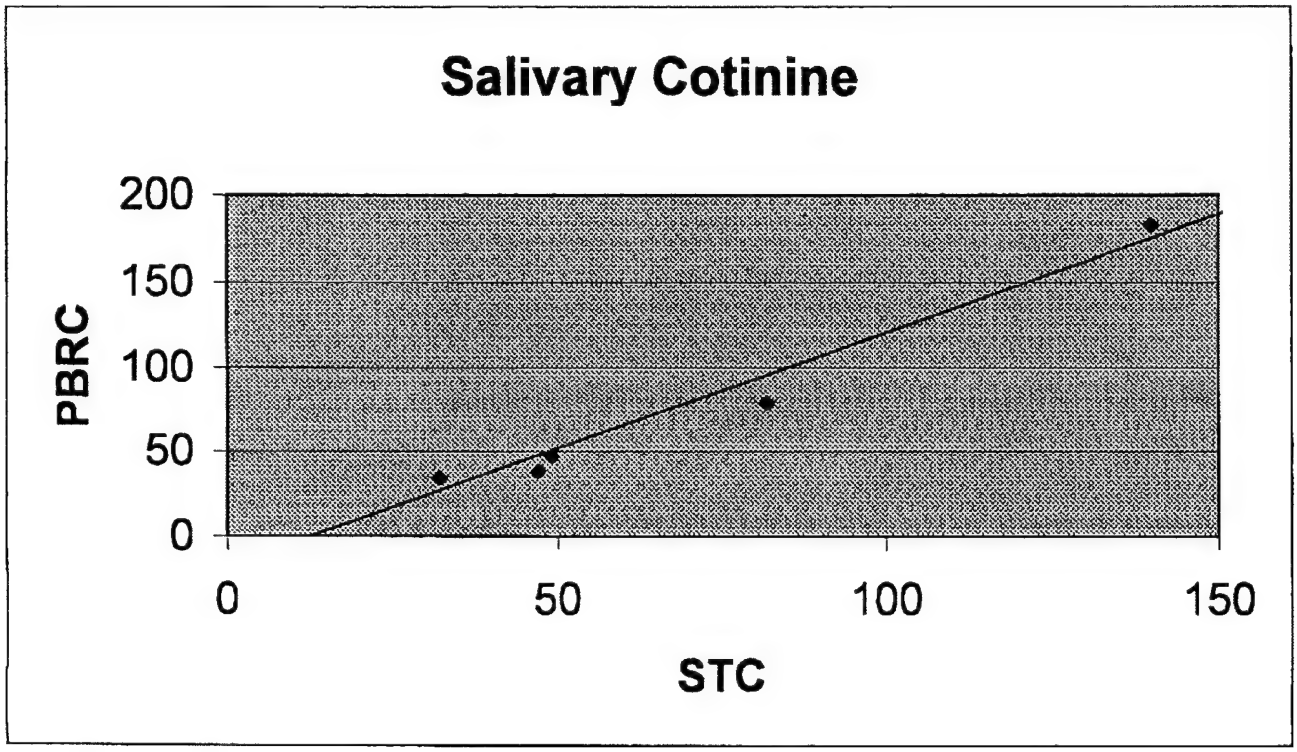
Bottle	
Conc of B:	50
rate 1	46
rate 2	48
Mean of B	47 rate

bottle conc	found rate
0	19
50	47

m	0.56
b	19
min det lim	2.9160592



	pbrc	stc
as	79	82
mh	47	49
bc	38	47
mb	34	32
rb	183	140



SUMMARY OUTPUT

Regression Statistics		
Multiple R	0.98614	
R Square	0.972471	0.98614
Adjusted	0.963295	
Standard	11.92871	
Observati	5	

ANOVA

	df	*SS*	*MS*	*F*	*gnificance F*
Regressio	1	15079.92	15079.92	105.9771	0.001955
Residual	3	426.8825	142.2942		
Total	4	15506.8			

	Coefficient	*andard Err*	*t Stat*	*P-value*	*ower 95%*	*pper 95%*	*ower 95.0*	*pper 95.0*
Intercept	-23.3374	11.043	-2.11332	0.124972	-58.4812	11.80638	-58.4812	11.80638

Enter data and click the following buttons to

IGF-2 ELISA (DSL)

Lab ID:	Pennington Biomedical Research Ctr.	CLIA Complexity:	Moderate
Analyte:	IGF-2	CDC Test System ID Code:	
Dose Unit:	NG/ML	CDC Analyte ID Code	
Lab Director:	Richard Tulley, PhD.	Lab Technologist:	JV

IGF-2
Intra-assay Precision Evaluation

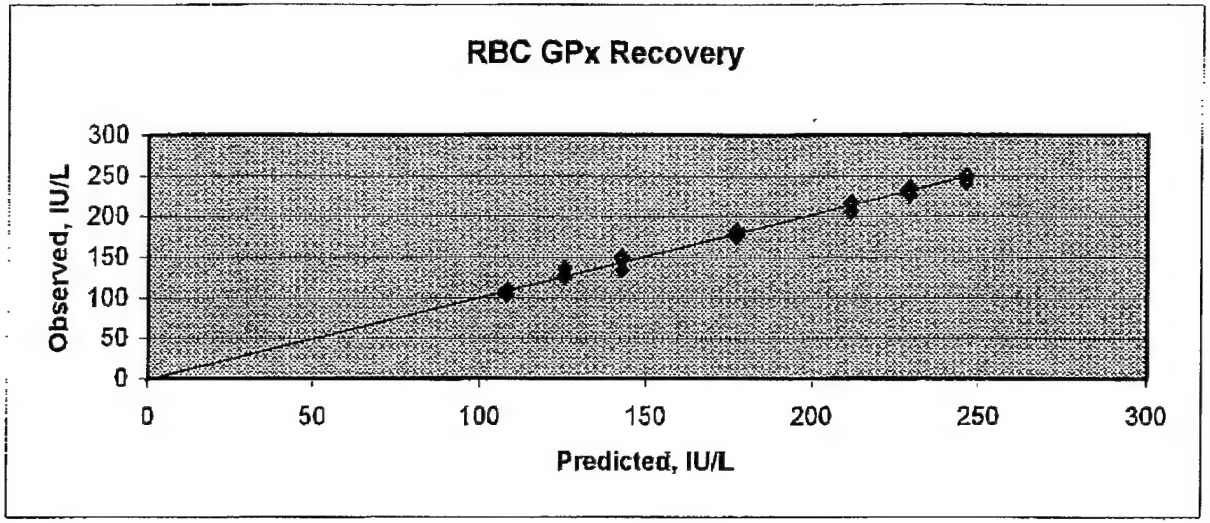
Replicate	Evaluation Samples							
				Low	High	Sample6	Sample7	Sample8
1				134.62	875.27			
2				112.39	725.52			
3				120.09	746.77			
4				102.99	856.97			
5				102.14	846.20			
6				124.36	761.95			
7				118.38	749.05			
8				102.99	840.13			
9				105.56	834.06			
10				120.09	736.15			
11				121.79	753.61			
12				114.96	877.01			
13				106.41	837.10			
14				123.50	733.11			
15								
16								
17								
18								
19								
20								
Mean				115.02	798.06			
SD				9.91	58.31			
CV				8.6%	7.3%			
N			0	14	14	0	0	0
PI claimed Conc.								
PI claimed CV								
Chi-square				#DIV/0!	#DIV/0!			
Crit. Chi-square				23.7	23.7			
Claim accepted				#DIV/0!	#DIV/0!			

Date(s) Performed: 4/18/00
Performance Specification:

Laboratory Criteria:

Acceptable: Y N

Reviewed & Approved by:
Signature/Date:



SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.9951066
R Square	0.9902372
Adjusted	0.9897233
Standard	5.1544457
Observati	21

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regressio	1	51201.32782	51201.33	1927.158	1.44E-20
Residual	19	504.7978982	26.56831		
Total	20	51706.12571			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*pper 95%*	*ower 95.0%*	*Upper 95.0%*
Intercept	3.0689921	4.160567692	0.737638	0.469747	-5.63918	11.77716	-5.6391788	11.77716311
X Variable	0.9934762	0.022630744	43.89941	1.44E-20	0.94611	1.040843	0.94610953	1.040842942

RBC GPx Recovery

	a	g
	109.6	246.1
	103.6	250.8
	110.6	241.3
ave	107.9333	246.0667

Sample	Low	Hi		predicted	Observed	Ave Obs.	Recovery	Ave. Rec.
a		200	0	107.9333	109.6		101.5442	
a		200	0	107.9333	103.6		95.98518	
a		200	0	107.9333	110.6	107.9	102.5	100.0
b		175	25	125.2	125.8		100.5	
b		175	25	125.2	136.3		108.9	
b		175	25	125.2	128.3	130.1	102.5	103.9
c		150	50	142.4667	147.3		103.4	
c		150	50	142.4667	134.2		94.2	
c		150	50	142.4667	150.9	144.1	105.9	101.2
d		100	100	177	175		98.9	
d		100	100	177	184		104.0	
d		100	100	177	179.3	179.4	101.3	101.4
e		50	150	211.5333	217.4		102.8	
e		50	150	211.5333	206		97.4	
e		50	150	211.5333	215.7	213.0	102.0	100.7
f		25	175	228.8	236.2		103.2	
f		25	175	228.8	232.4		101.6	
f		25	175	228.8	226.4	231.7	99.0	101.3
g		0	200	246.0667	246.1		100.0	
g		0	200	246.0667	250.8		101.9	
g		0	200	246.0667	241.3	246.1	98.1	100.0
							Ave	101.2
							SD	1.3332

APPENDIX

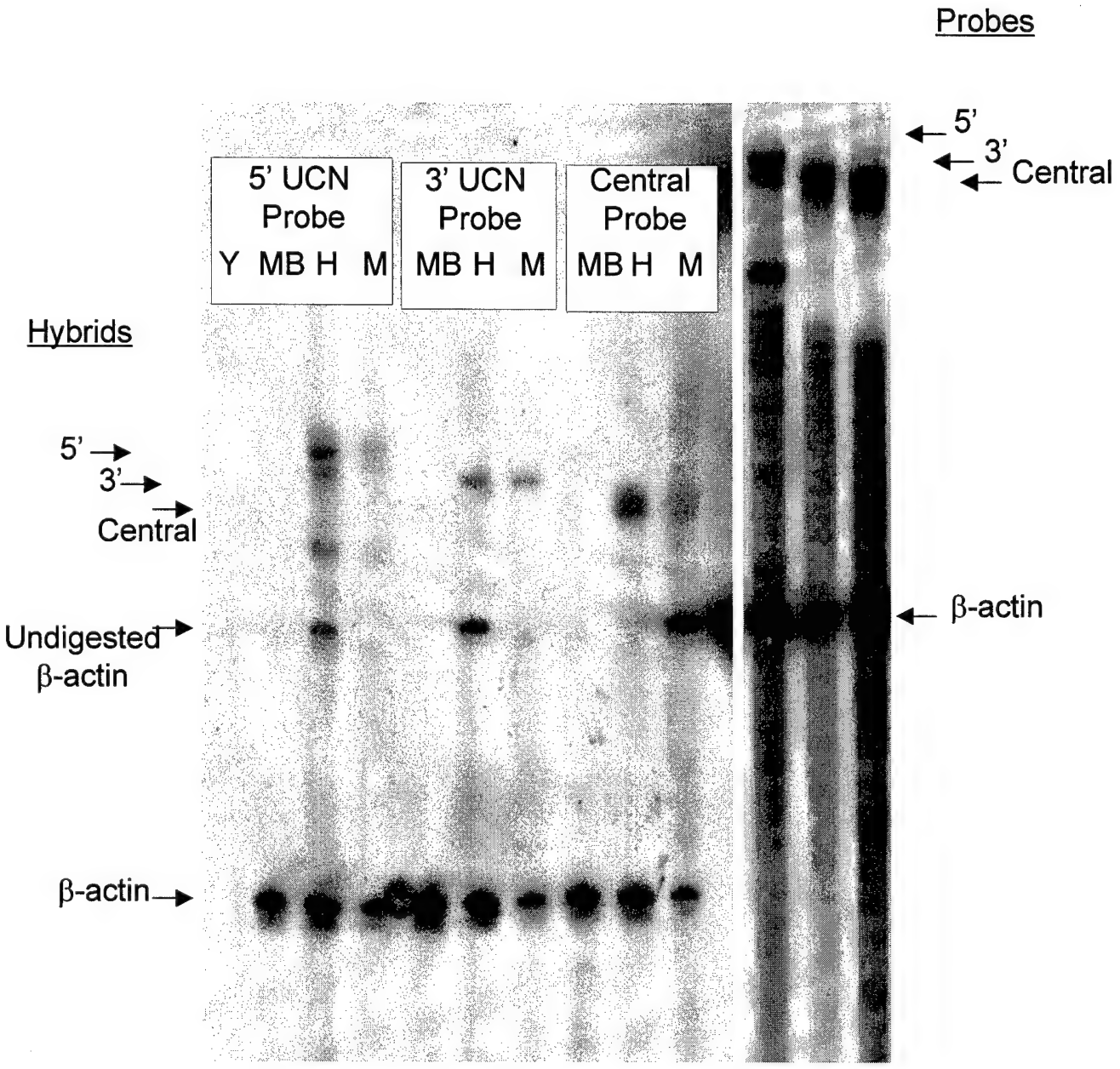
TASK II: STABLE ISOTOPE LABORATORY

NONE

APPENDIX

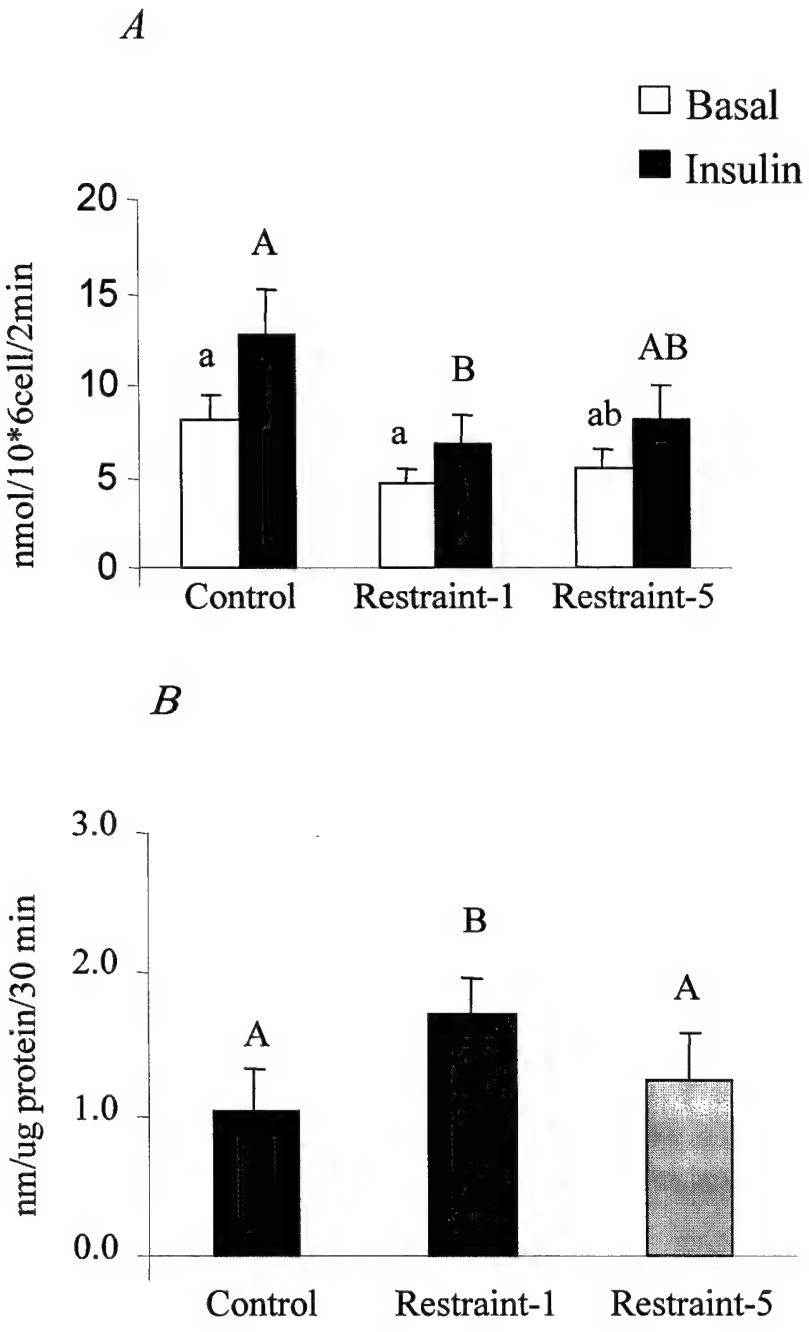
TASK III: STRESS, NUTRITION AND MENTAL PERFORMANCE

Figure 1



Detection of antisense UCN RNA in an RPA using 20 ug of total RNA from tissues from restrained rats and three different sense UCN riboprobes, spanning almost the full length of UCN (bases 1 to 560). Protected fragments of antisense UCN and of β– actin are indicated by arrows. Y – yeast, MB -midbrain, H - heart, M – skeletal muscle.

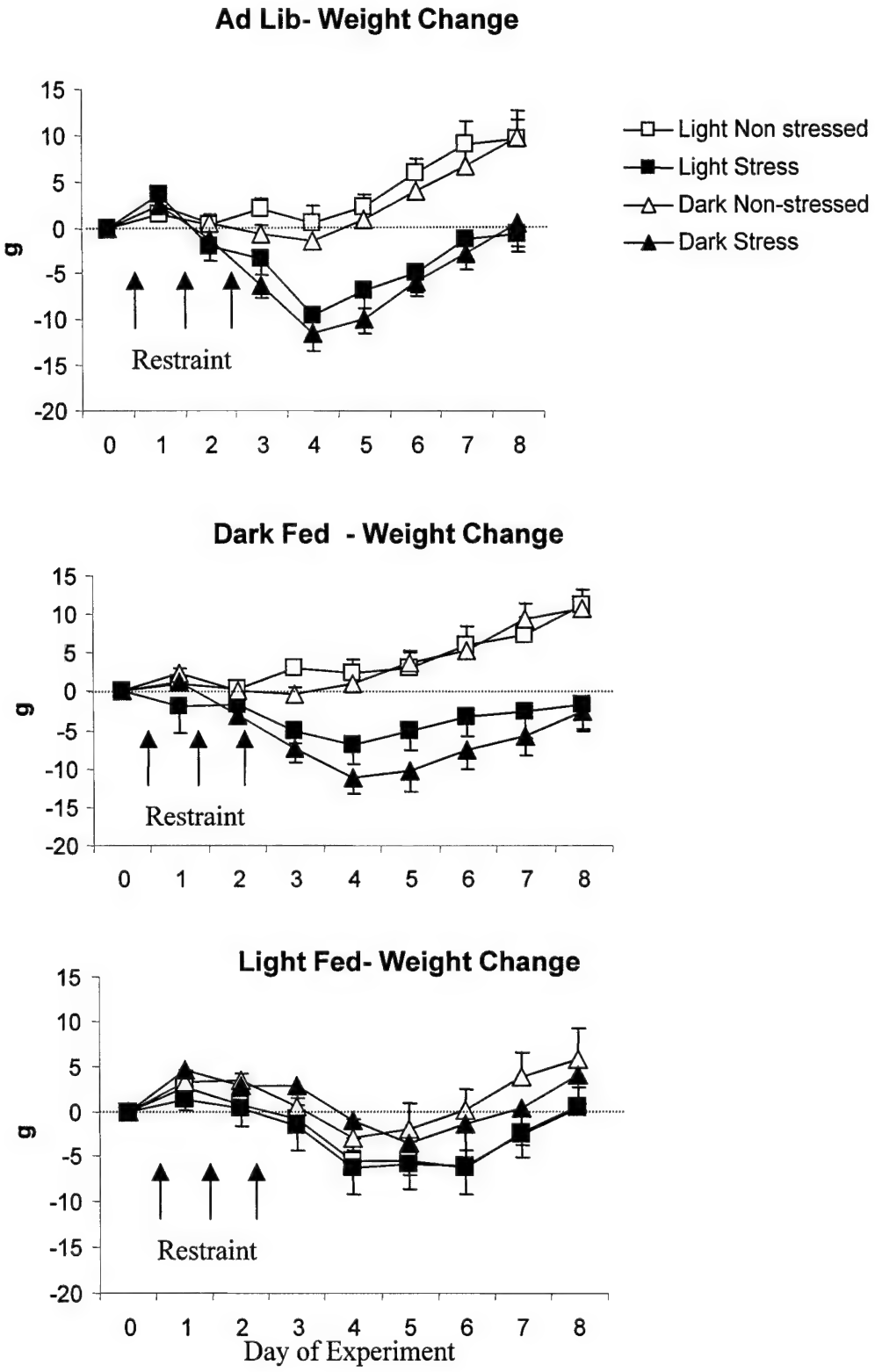
Figure 2



A: Adipocyte glucose transport in control, Restraint-1, and Restraint-5 groups of rats Data are means + SEM for groups of 10 rats. Insulin significantly increased glucose transport in all three groups of rats ($P<0.05$), but glucose transport was significantly reduced in Restraint-1 rat compared with control rats ($P<0.05$). Restraint-5 rats were not different from either control or Restraint-1 groups.

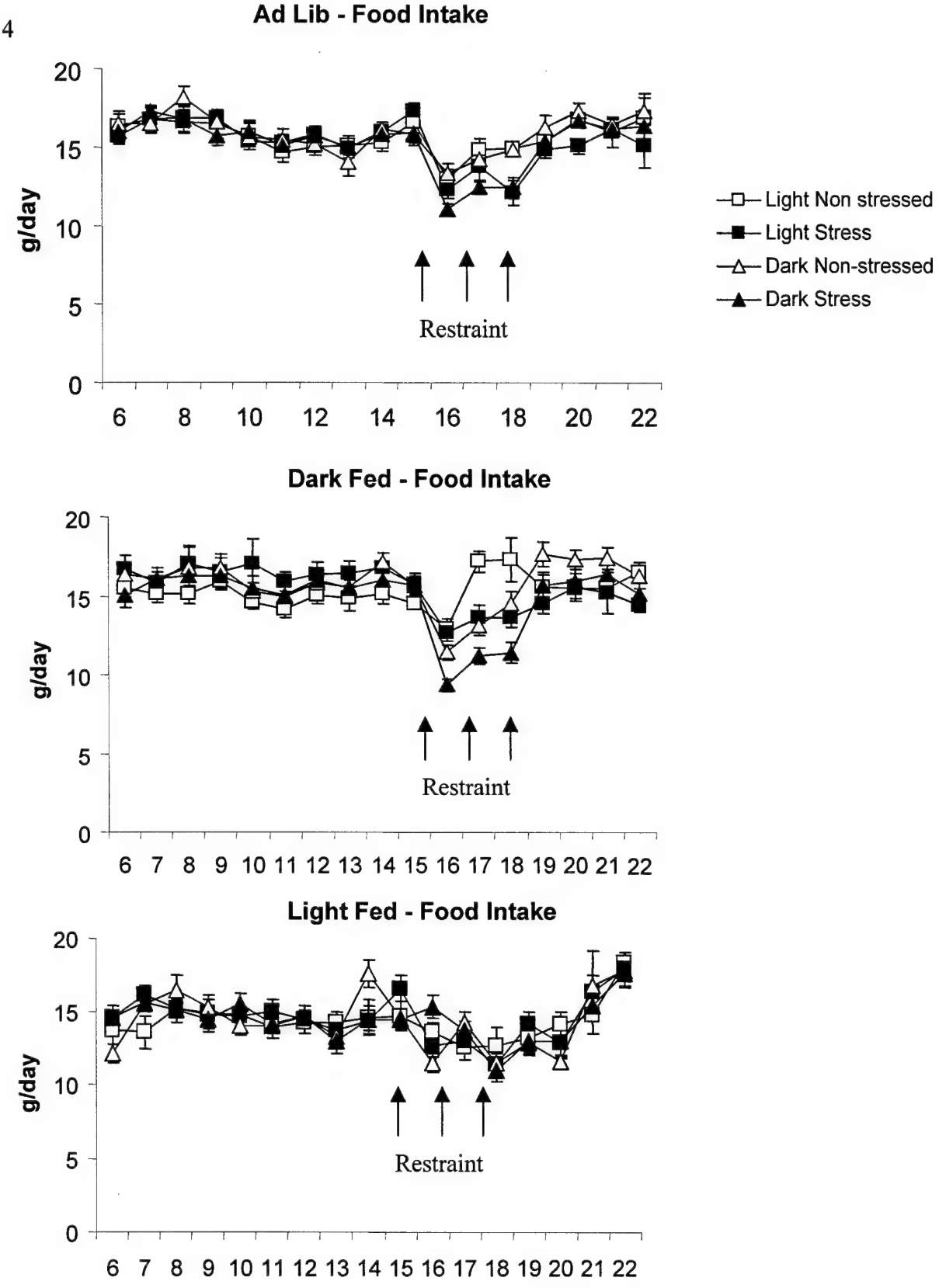
B: β -adrenergic receptor binding of ^{3}H-DHA in control, Restraint-1, and Restraint-5 groups of rats. Data are means ± SEM for groups of 10 rats. β–adrenergic ligand binding was significantly higher for Restraint-1 than control or Restraint-5 animals ($P< 0.05$). There was no difference between control and Restraint –5 rats.

Figure 3



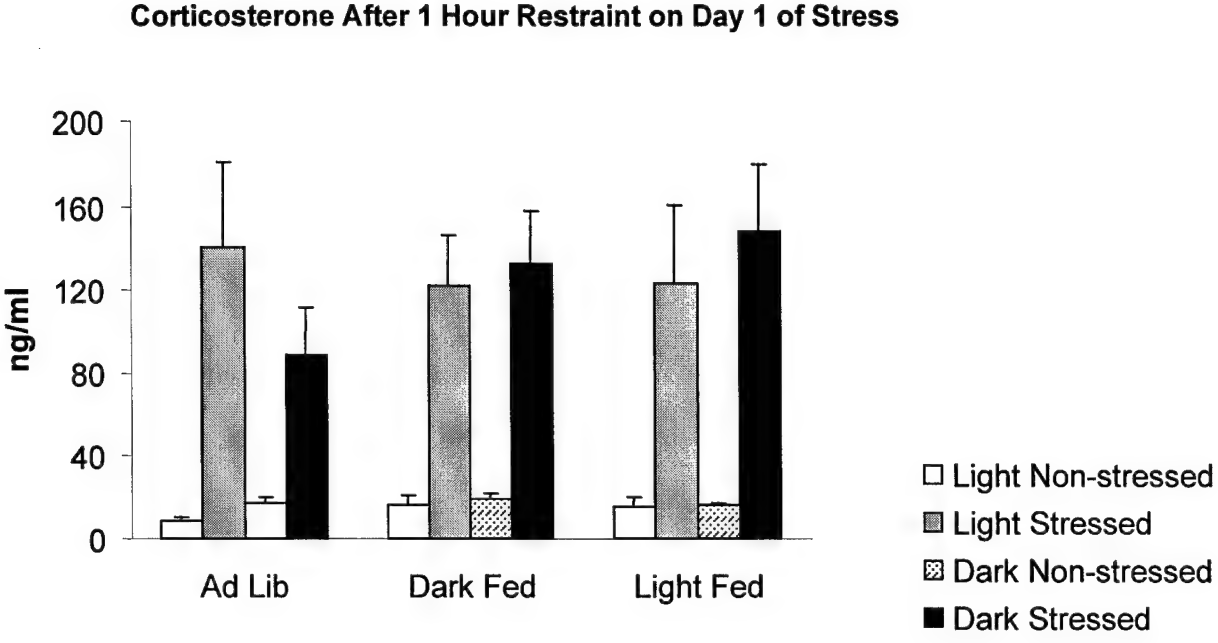
Weight change in rats exposed to 3 hours restraint stress on each of days 1 to 3. Data are means $\pm$ sem for groups of 8 rats. There were three feeding treatments, Ad lib, Dark fed and Light fed, and within each of these treatments there were four groups: light stressed, light non-stressed, dark stressed and dark non-stressed.

Figure 4



Daily food intake of rats exposed to 3 hours restraint stress on each of days 15 to 17. Data are means ± sem for groups of 8 rats. There were three feeding treatments, Ad lib, Dark fed and Light fed, and within each of these treatments there were four groups: light stressed, light non-stressed, dark stressed and dark non-stressed.

Figure 5


Corticosterone After 1 Hour Restraint on Day 1 of Stress
200
160
120
80
40
0
ng/ml
Ad Lib
Dark Fed
Light Fed
Light Non-stressed
Light Stressed
Dark Non-stressed
Dark Stressed

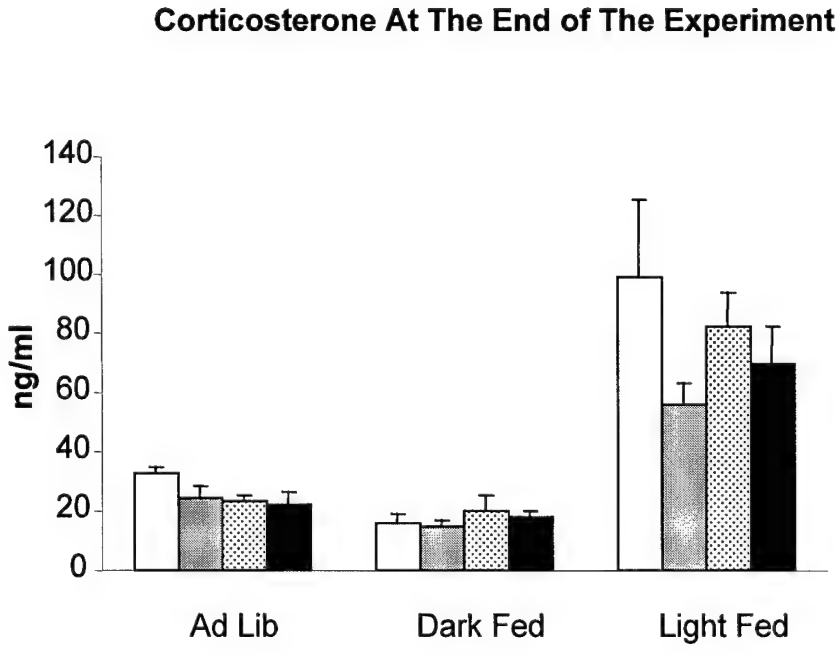
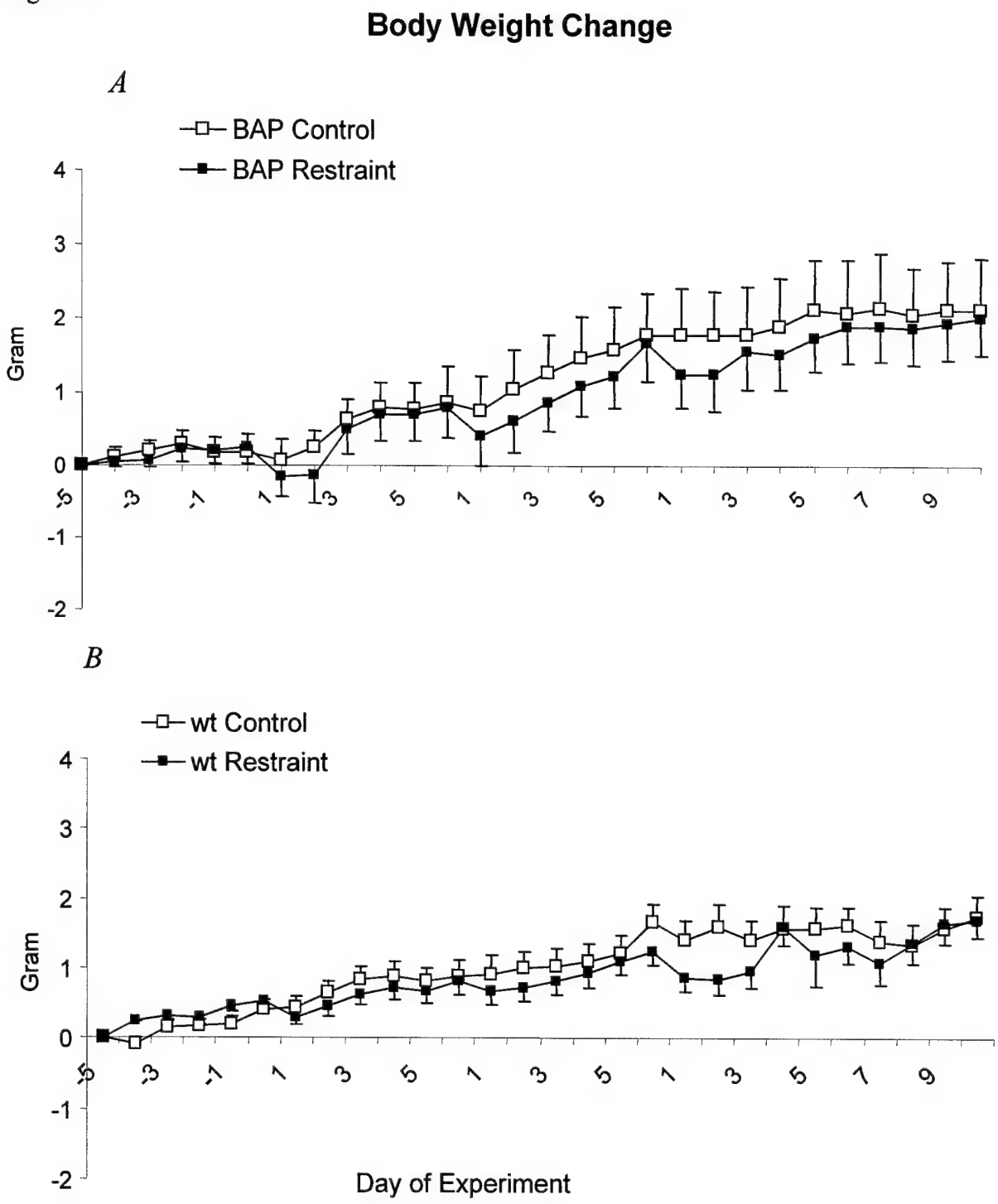
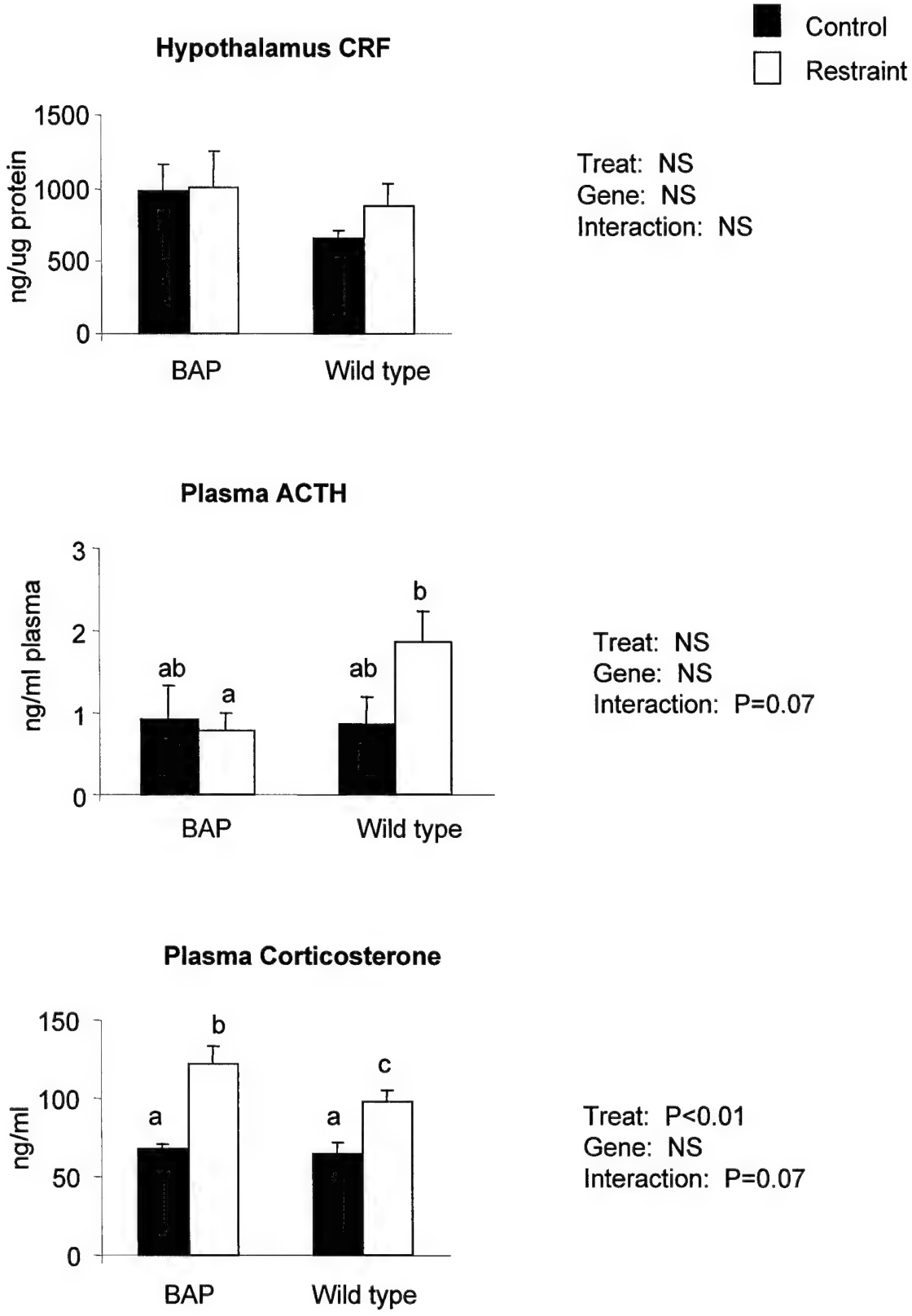

Corticosterone At The End of The Experiment
140
120
100
80
60
40
20
0
ng/ml
Ad Lib
Dark Fed
Light Fed

Figure 6



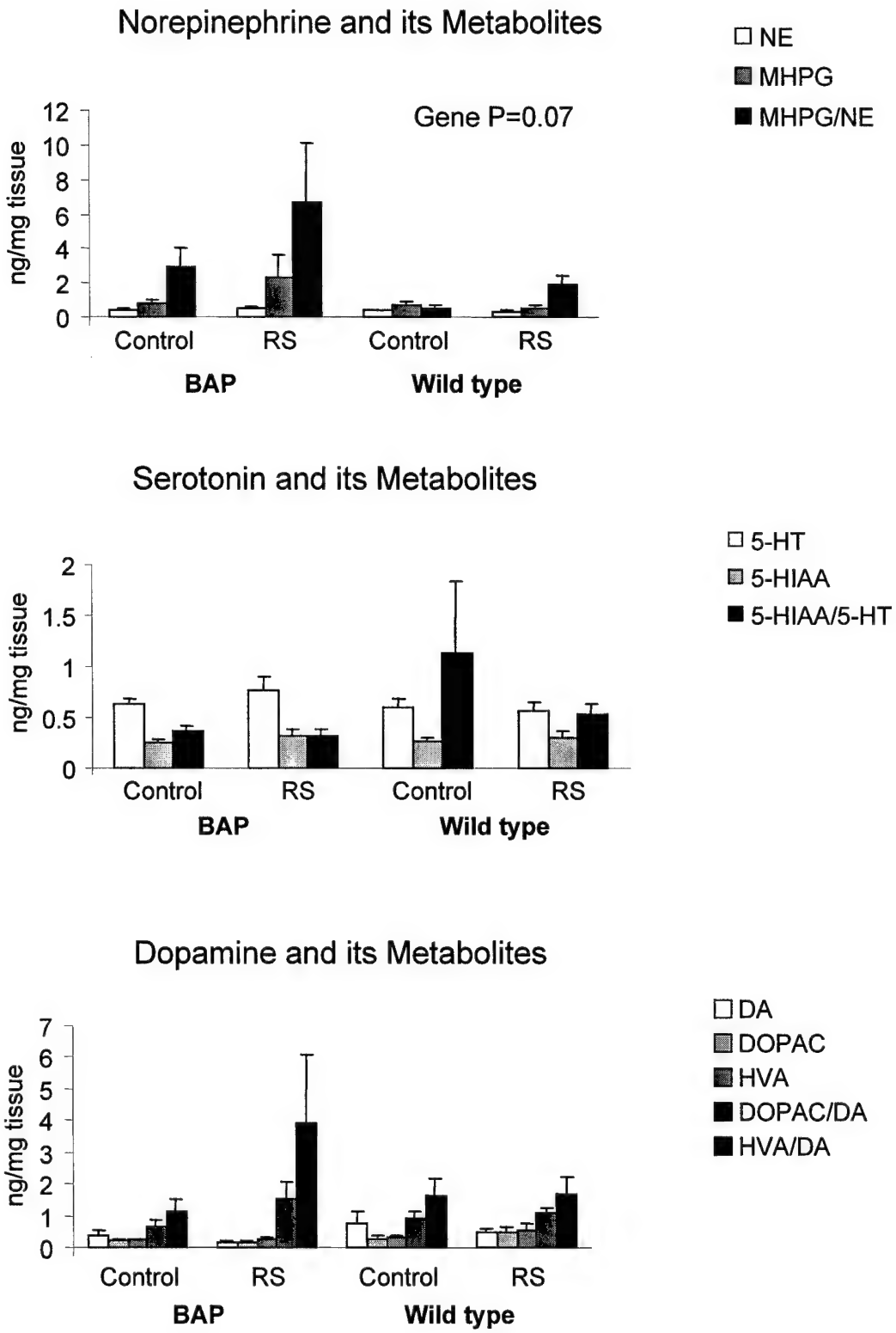
Body weight change in BAP (A) and wild type (B) mice exposed to 12 minutes of restraint stress at three times during the experiment. There was no effect of stress on weight gain in either genotype.

Figure 7



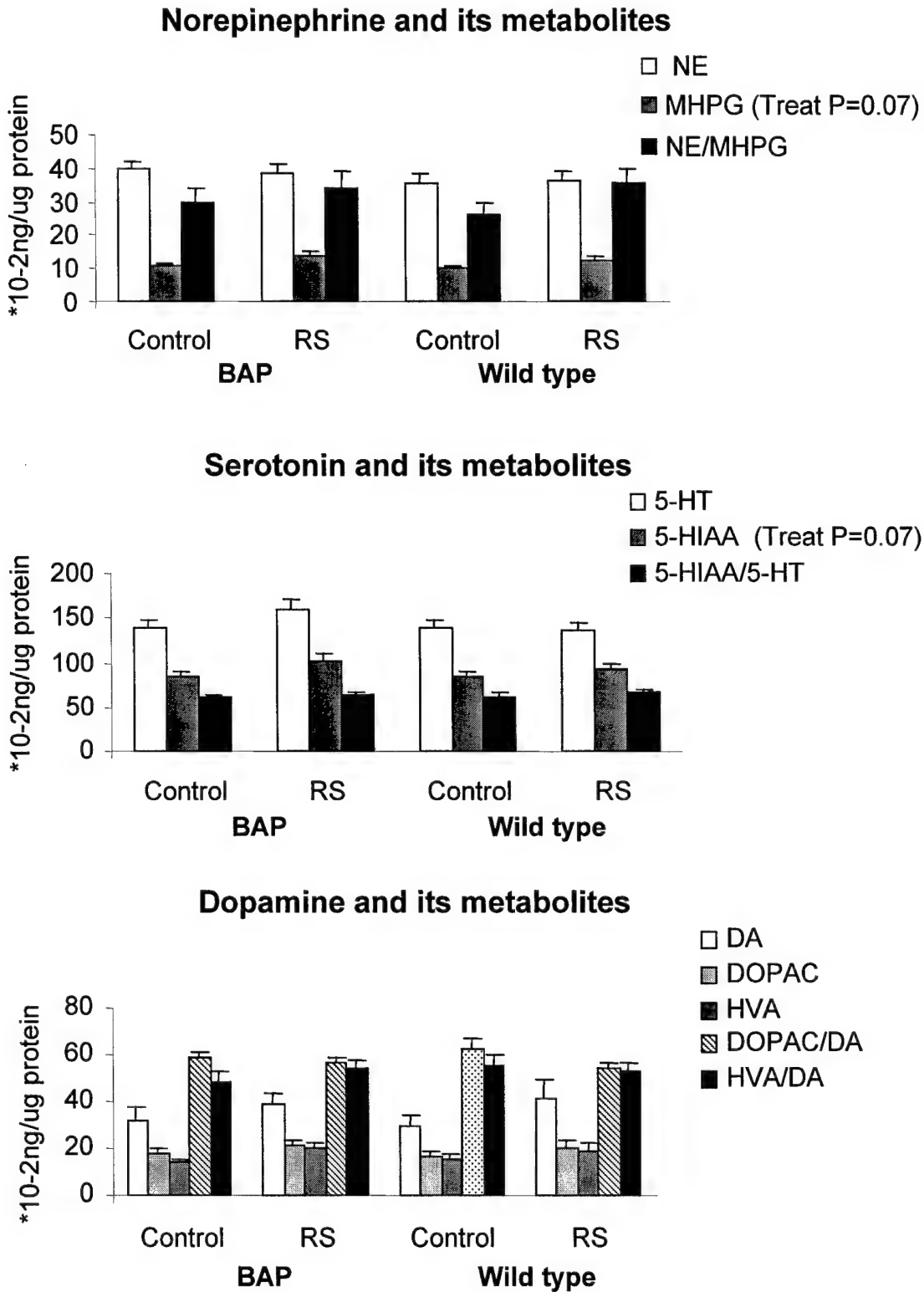
Measures of HPA axis activity measured after 30 minutes of restraint in mice over-expressing agouti protein (BAP) or wild type mice. Data are means + sem for groups of 10 animals. Values that do not share a common superscript are significantly different at $P<0.05$.

Figure 8:



Frontal cortex monoamines measured after 30 minutes of restraint in mice over-expressing agouti protein (BAP) or wild type mice. Data are means + sem for groups of 10 animals.

Figure 9

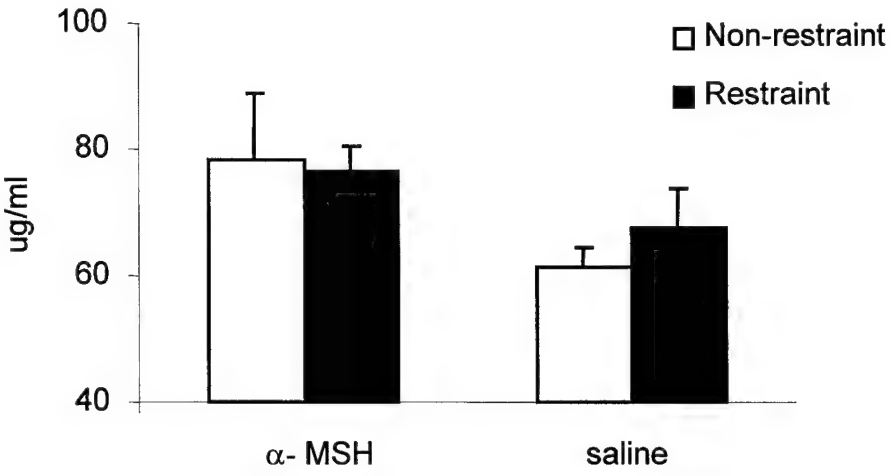


Data are means + sem for groups of 12 mice. The mice were killed at the end of a 12 minute restraint stress, the amygdala was dissected and snap frozen prior to HPLC analysis of catecholamines and their metabolites.

Figure 10

Plasma Corticosterone Measured after 30 minutes of the First Restraint

α-MSH vs. Saline: P=0.06
Non-restraint vs. Restraint: NS
Interaction: NS

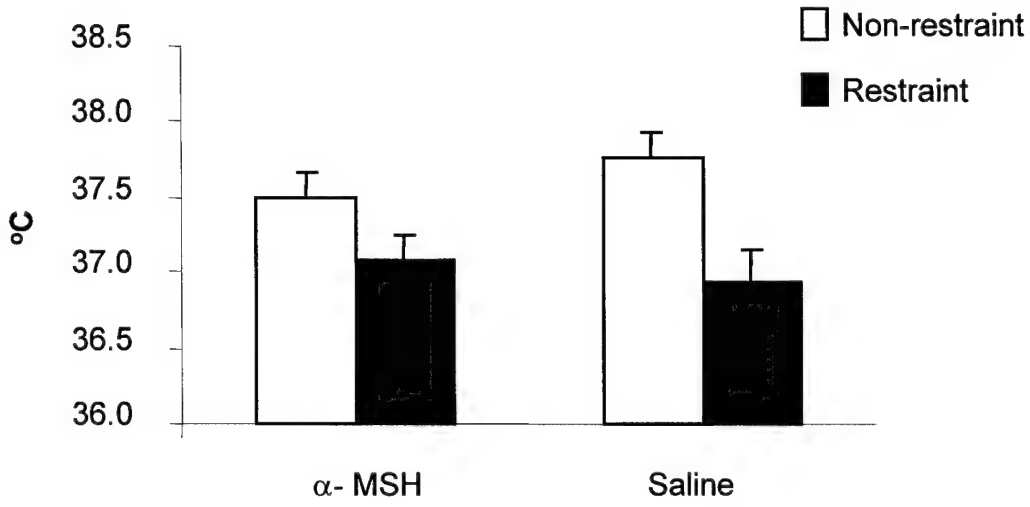


Data are means + sem for groups of 10 C57BL/6J mice. The mice received an i.p. injection of either saline or 160 ug αMSH 30 minutes before the start of restraint. Blood was collected by tail bleeding after 30 minutes of the first of 3 daily 2 hour periods of restraint.

Figure 11

Rectal Temperature Measured at the End of the Third Restraint

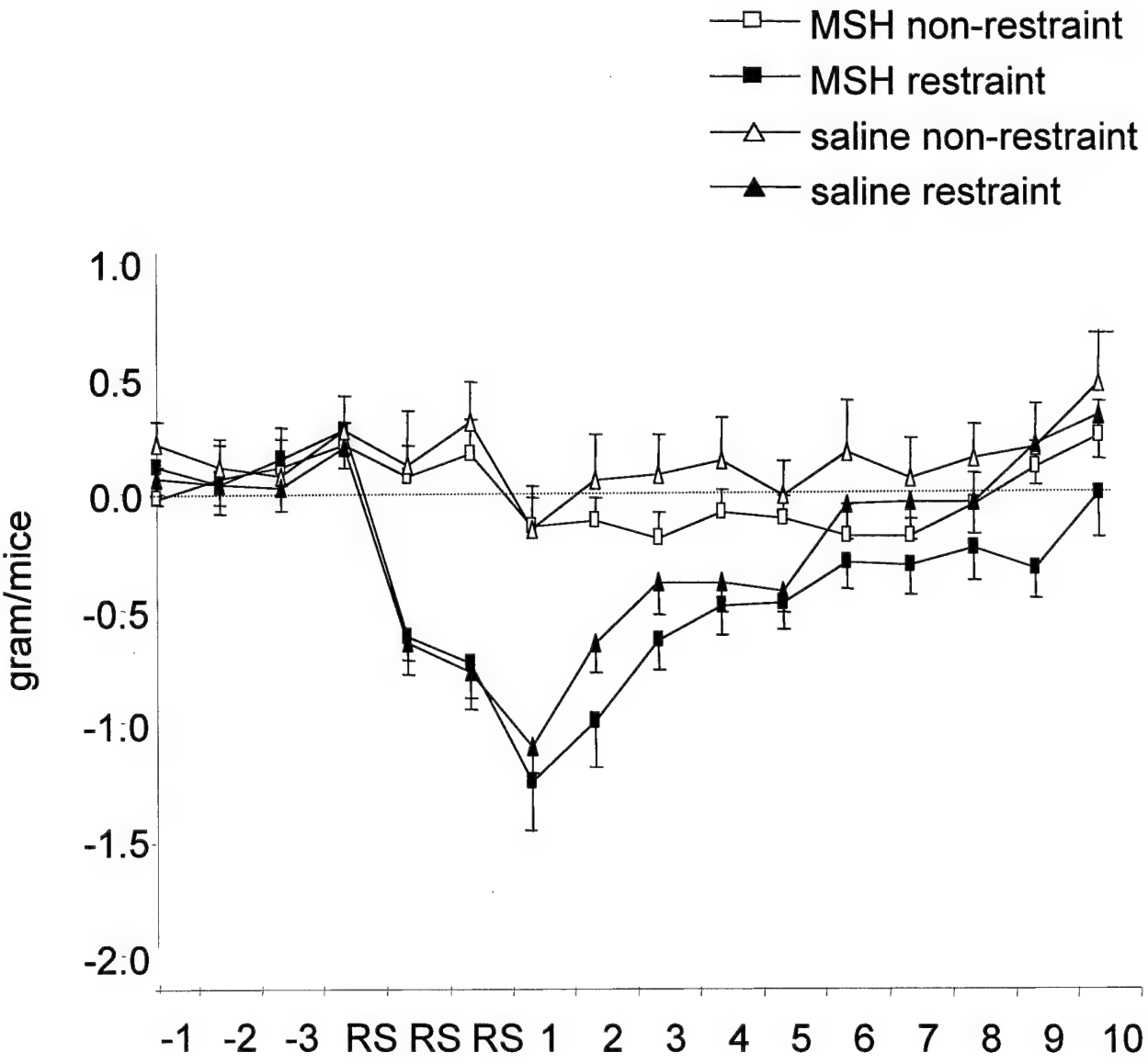
α-MSH vs.. Saline: NS
Non-restraint vs.Restraint: P<0.01
Interaction: NS



Data are means + sem for groups of 10 C57BL/6J mice. The mice received an i.p. injection of either saline or 160 ug α–MSH 30 minutes before the start of a 2 hour restraint. Rectal temperature was measured at the end of the third of 3 daily periods of restraint.

Figure 12

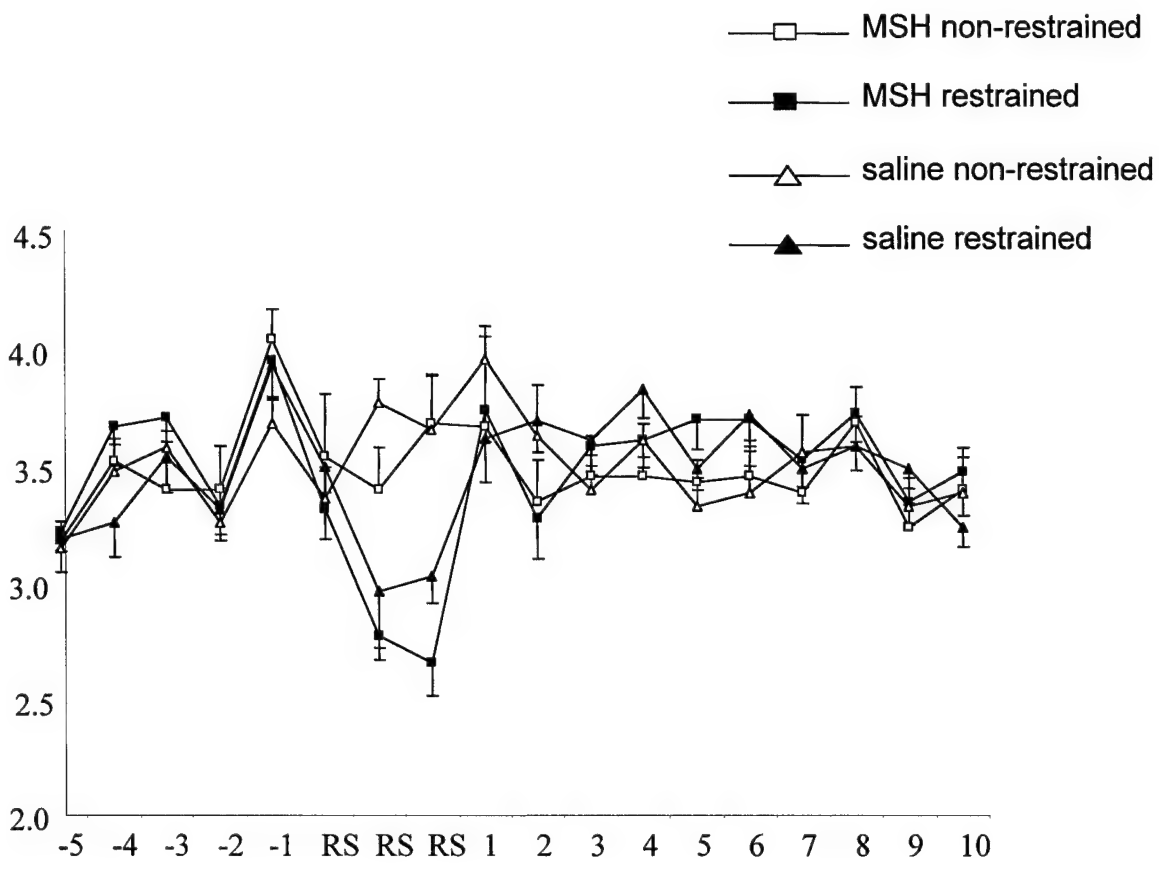
Body Weight Change In Mice Exposed to Repeated Restraint



Data are means + sem for groups of 10 C57BL/6J mice. The mice received an i.p. injection of either saline or 160 ug αMSH 30 minutes before the start of a 2 hour restraint on each of the 3 days marked RS.

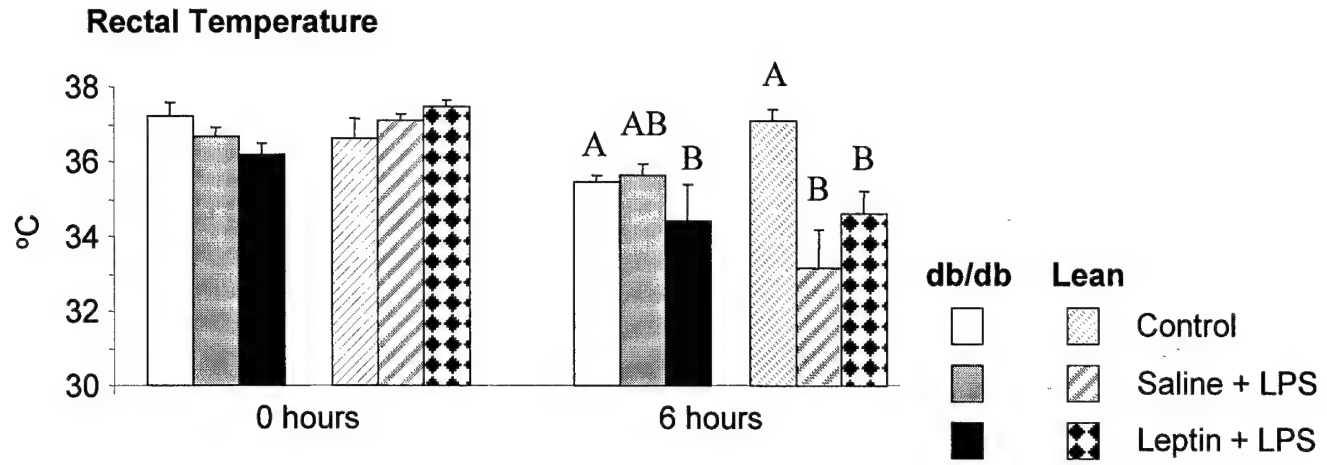
Figure 13

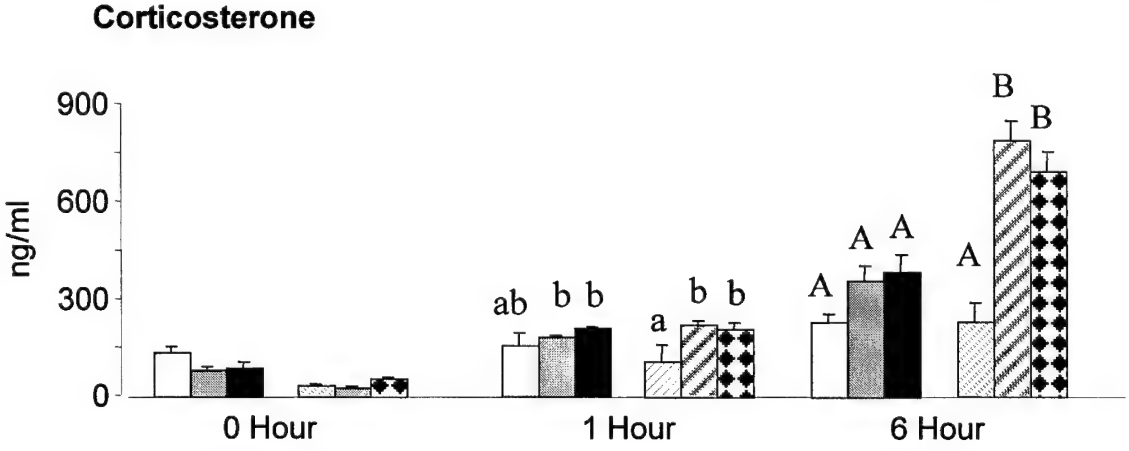
Food Intakes of Mice Exposed to Repeated Restraint Stress

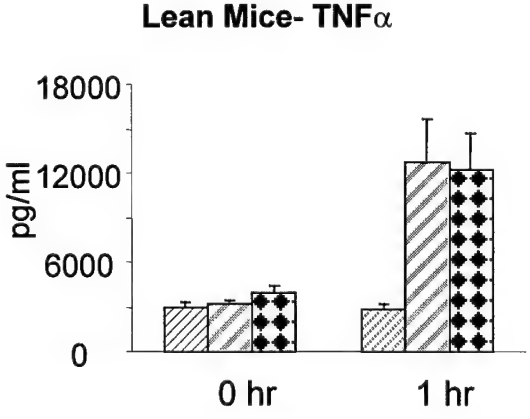


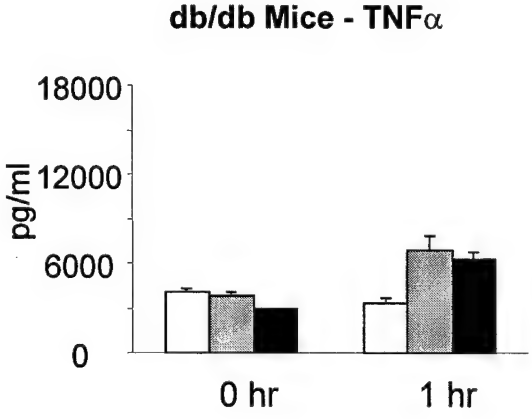
Data are means + sem for groups of 10 C57BL/6J mice. The mice received an i.p. injection of either saline or 160 ug αMSH 30 minutes before the start of a 2 hour restraint on each of the 3 days marked RS.

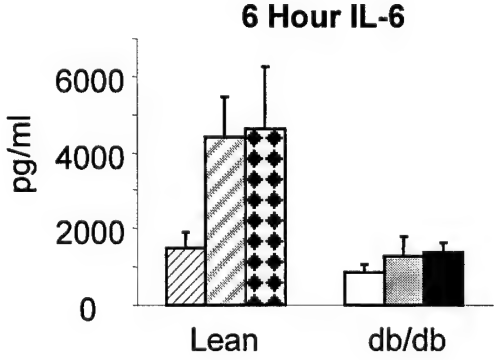
Figure 14





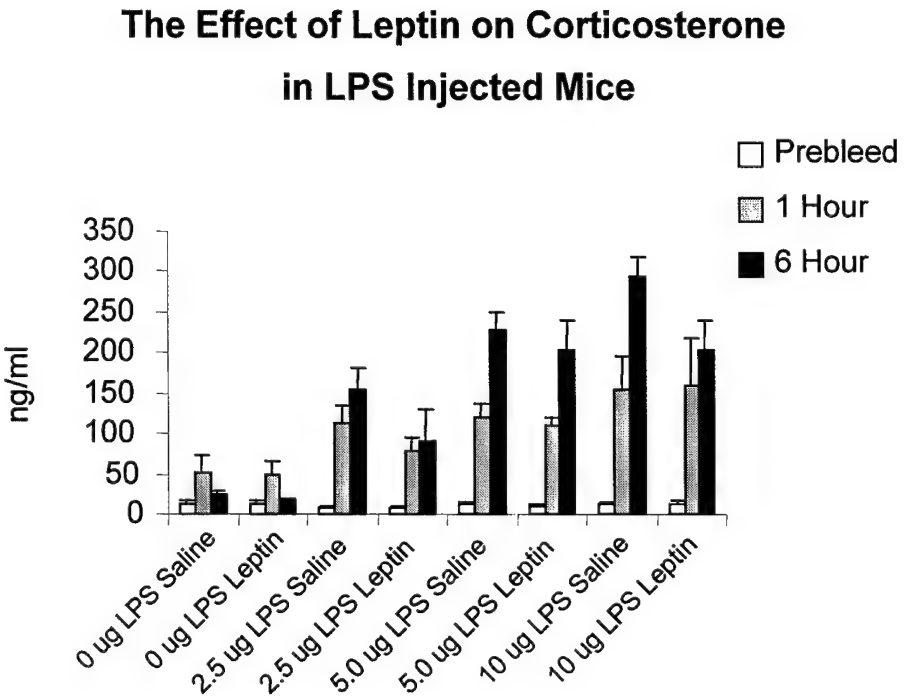






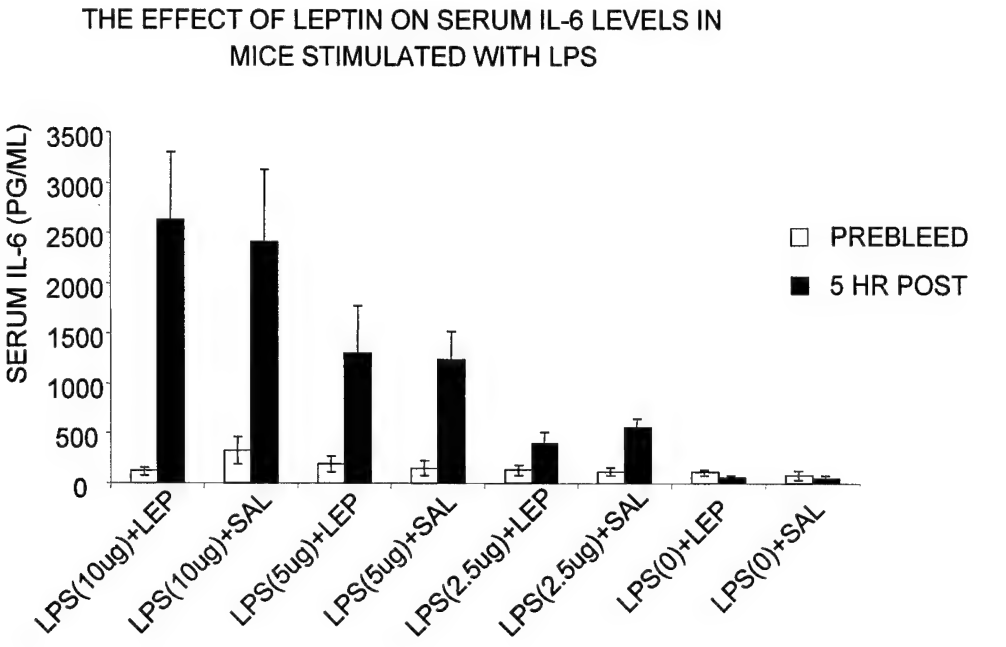
Rectal temperature and serum cytokines in lean and obese (db/db mice injected with LPS and leptin. There was no effect of leptin on cytokine production

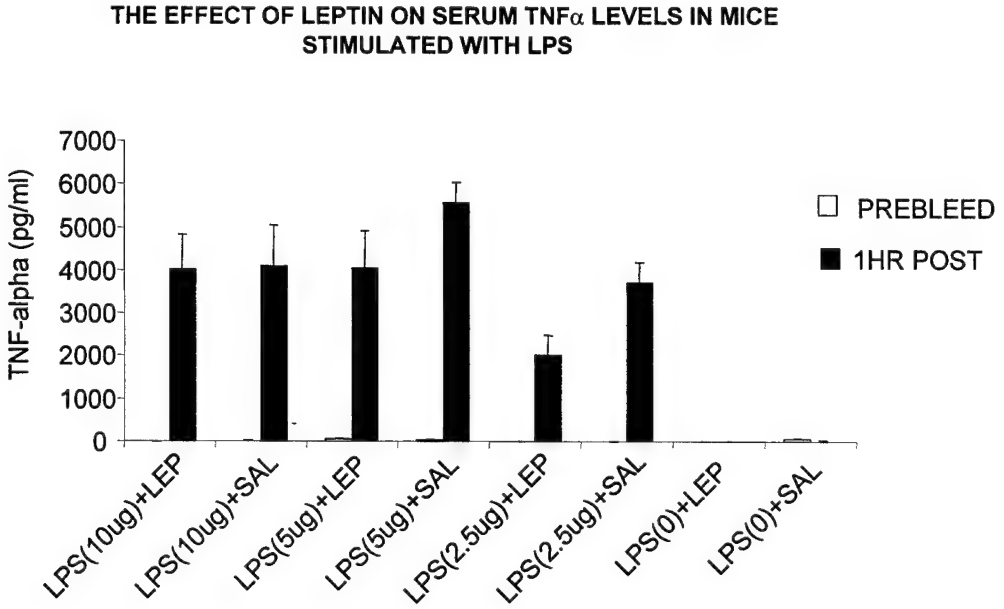
Figure 15



Serum corticosterone concentrations measured at different time intervals after LPS and leptin or saline injection. Leptin inhibited LPS-induced corticosterone release five hours after LPS injection.

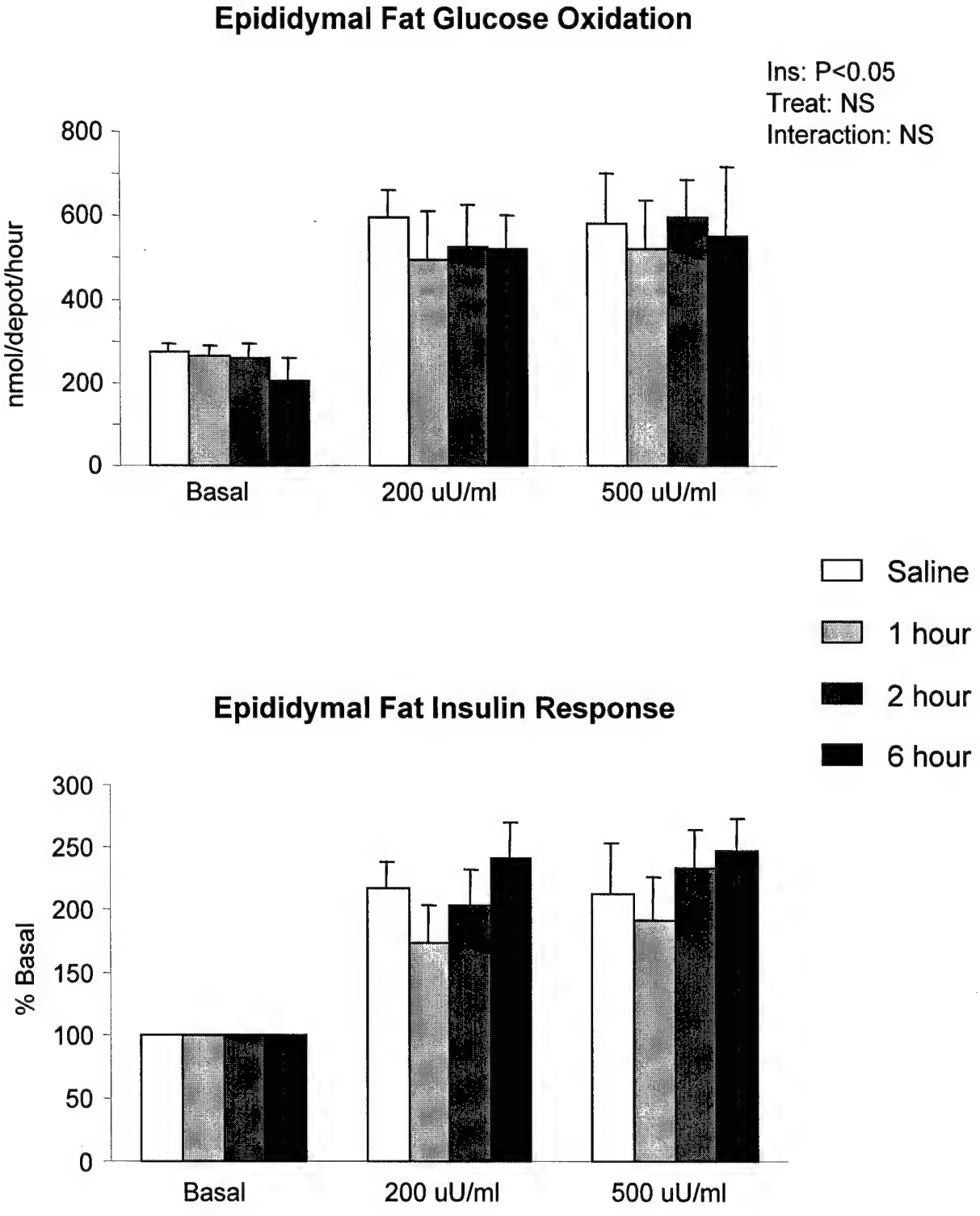
Figure 16:





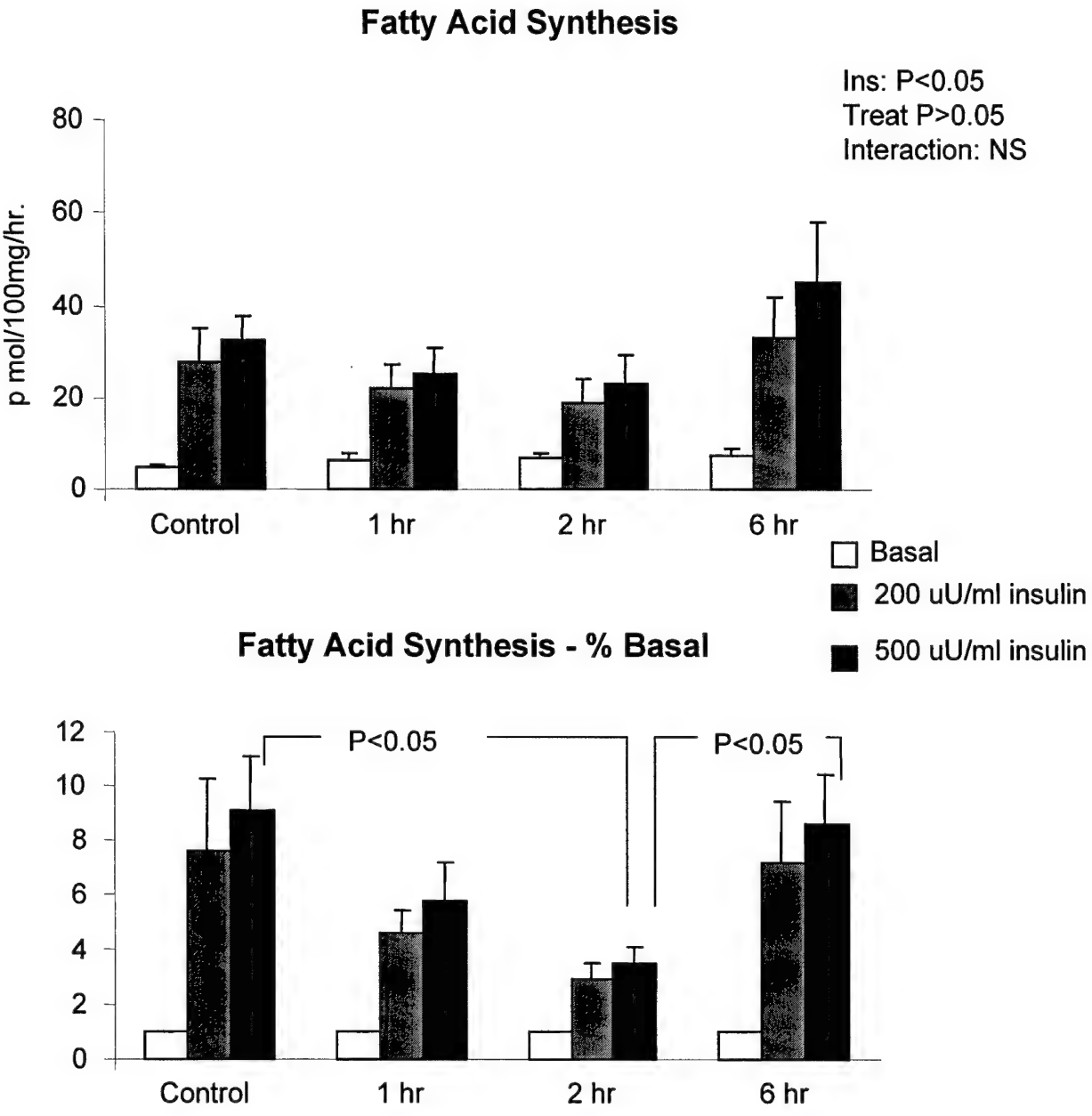
Serum TNFα and IL-6 measured one and five hours, respectively, after LPS injection. LPS stimulated release of both cytokines but leptin had no effect on the response to LPS.

Figure 17:



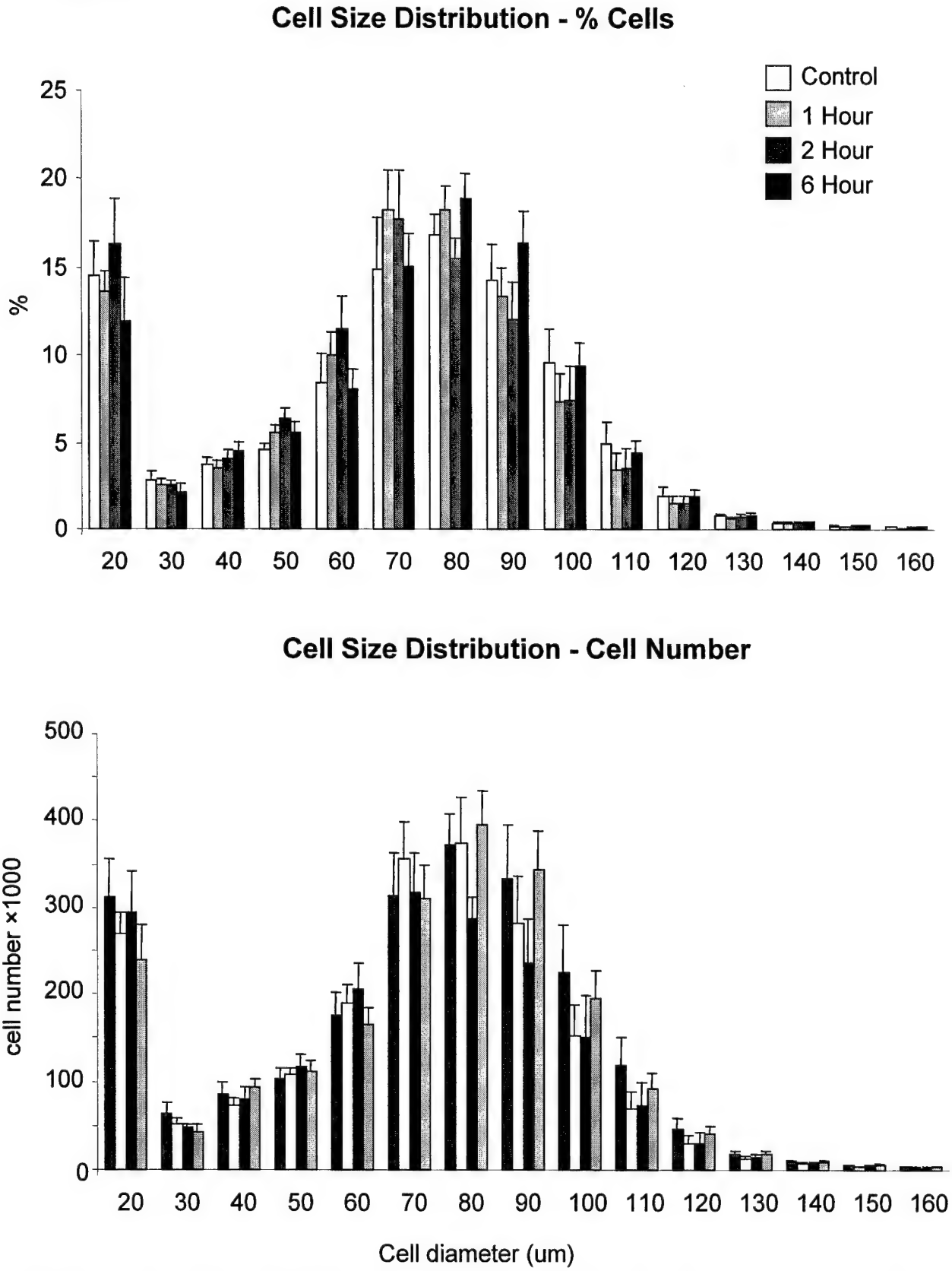
Glucose oxidation in adipose tissue from rats injected with LPS at timed intervals prior to sacrifice. Insulin significantly increased oxidation but there was no effect of LPS on the rate of oxidation.

Figure 18



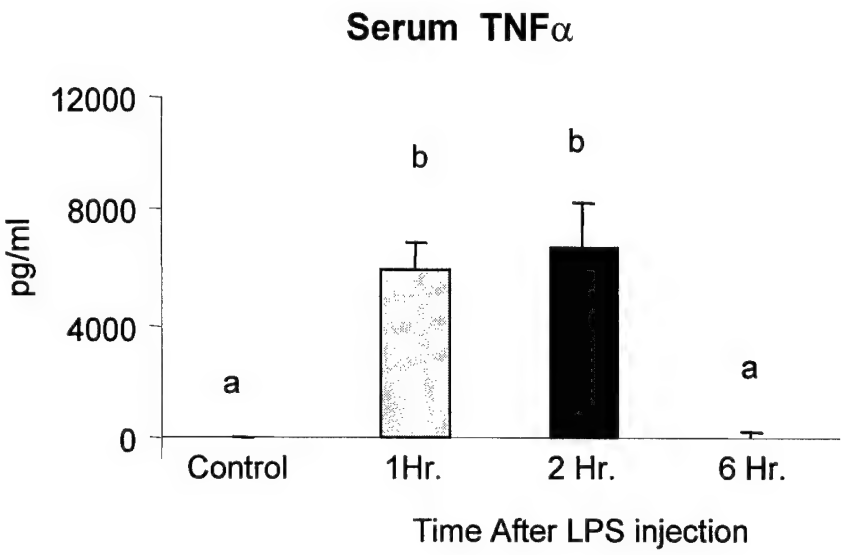
: Glucose incorporation into lipids in adipose tissue from rats injected with LPS at timed intervals prior to sacrifice. Insulin significantly increased oxidation and LPS significantly inhibited this response 2 hours after LPS injection

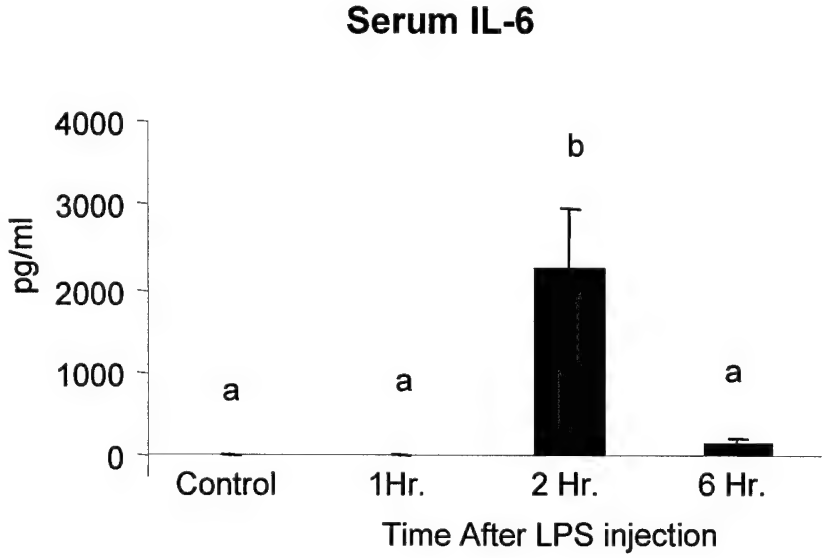
Figure 19:



Epididymal fat cell size distribution in rats treated with LPS at timed intervals prior to sacrifice. There was a reduction in the number of large cells present 2 hours after injection.

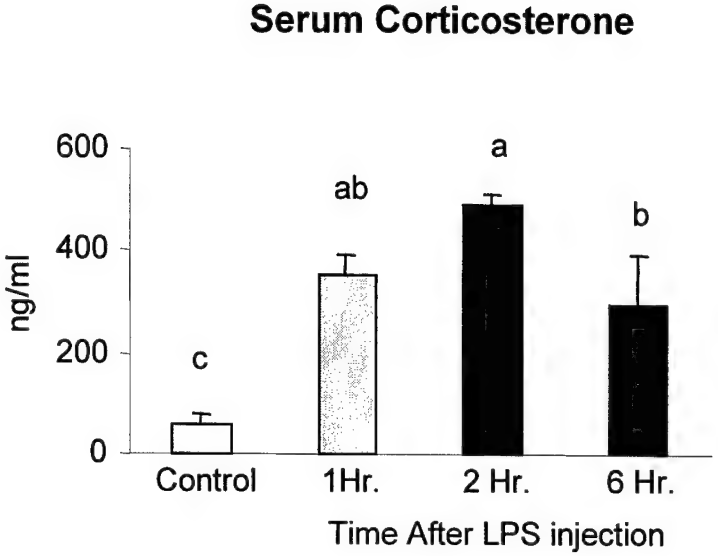
Figure 20

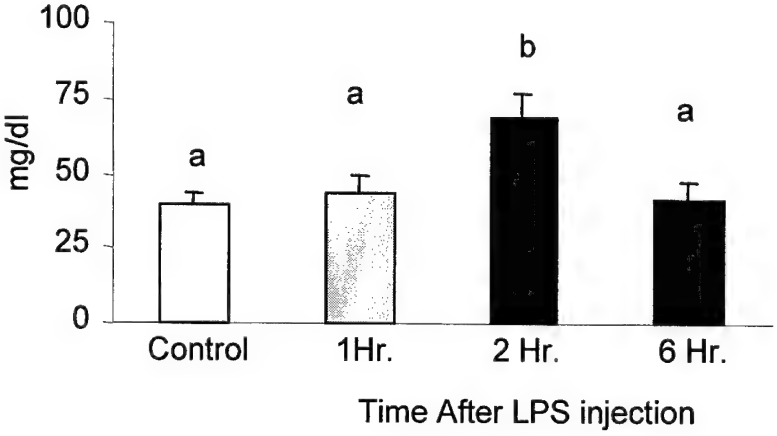




Serum TNFα and IL-6 measured in rats treated with LPS at timed intervals prior to sacrifice. Values that do not share a common superscript are significantly different at $P<0.05$.

Figure21:

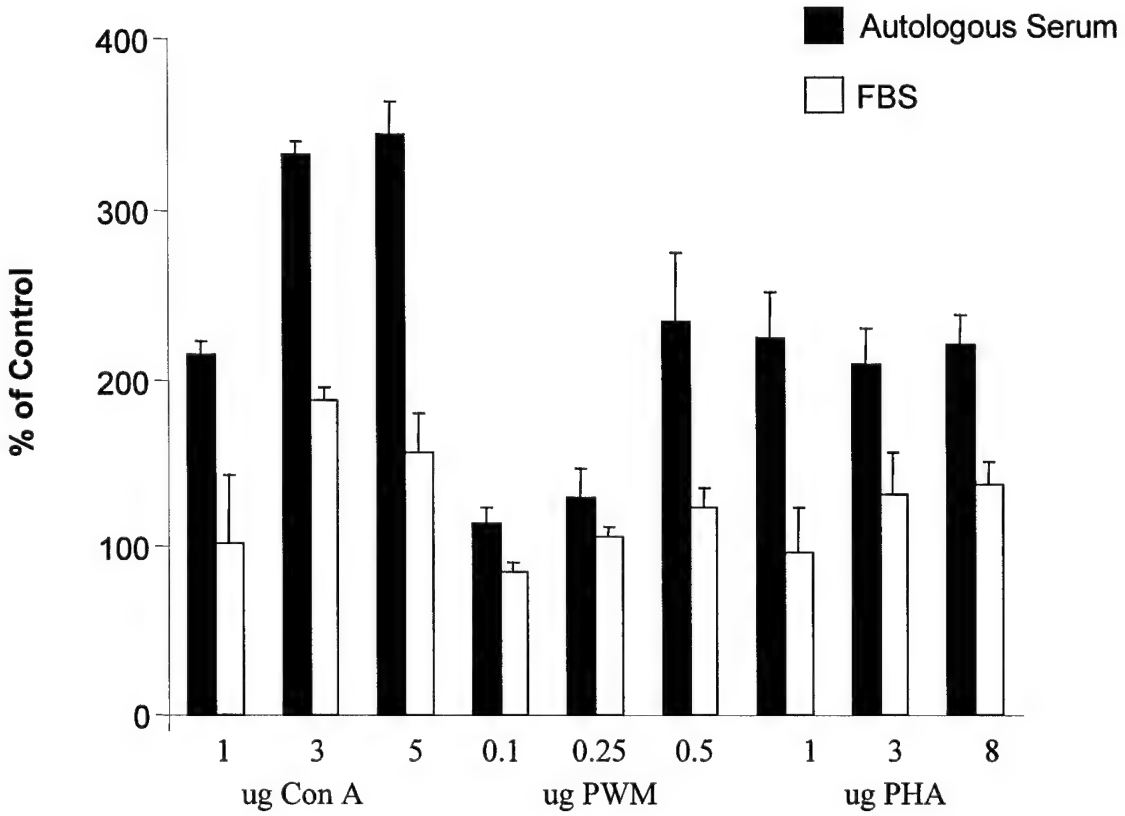




Serum corticosterone and triglycerides measured in rats treated with LPS at timed intervals prior to sacrifice. Values that do not share a common superscript are significantly different at $P<0.05$.

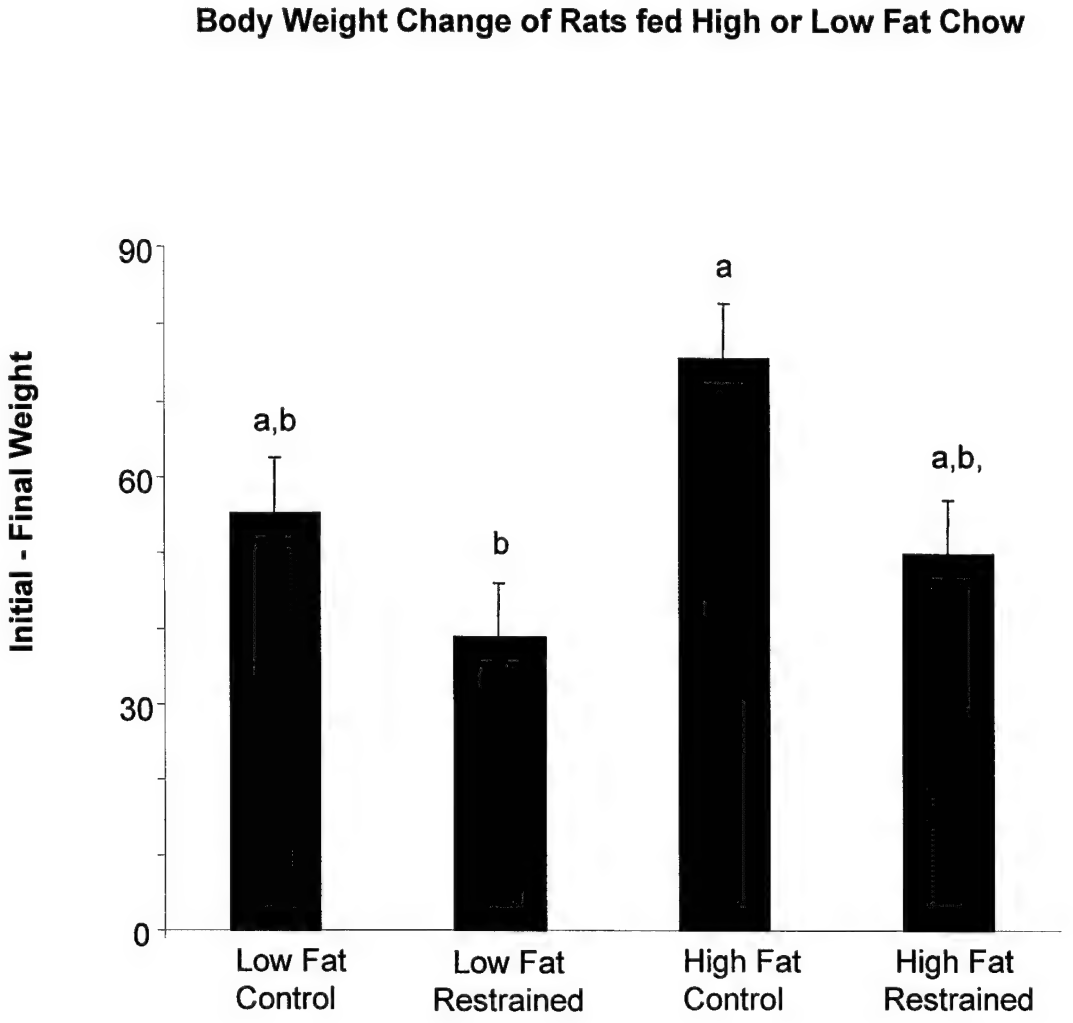
Figure 22

Comparison of Proliferation in the Presence of FBS and Autologous Rat Serum



Data are means + sem for groups of 3 rats fed chow. Proliferation was measured in the presence of 10 % autologous serum or 10% fetal bovine serum (FBS)

Figure 23



Data are means + sem for groups of 4 rats. Values that do not share a common superscript are significantly different at $P<0.05$

APPENDIX

TASK IV: STRESS, NUTRITION AND WORK PERFORMANCE

Table 1.

Age	Weight (kg)	Height (cm)	% Fat	Body Mass Index (BMI)	VO_2 (ml/kg/min)
26.2 ± 2.1	68.8 ± 2.8	177.4 ± 2.8	13.2 ± 1.1	21.8 ± 0.6	69.2 ± 3.3

Data are mean ± SE

Table 2. Bout 1 Blood Data

	CHO	BCAA	EAA
Glucose (mg/dL)			
Pre	95 ± 2	93 ± 3	94 ± 2
Fatigue	90 ± 8	88 ± 4	92 ± 6
FFA (mM)			
Pre	0.187 ± 0.023	0.200 ± 0.033	0.237 ± 0.032
Fatigue	0.514 ± 0.140[a]	0.438 ± 0.042[a]	0.462 ± 0.063[a]
Prolactin (ng/mL)			
Pre	7.47 ± 1.05	7.37 ± 0.83	7.06 ± 0.44
Fatigue	9.69 ± 1.13	7.08 ± 0.78[b]	8.71 ± 0.94

Data are mean ± SE. [a] significant time effect $p < 0.01$, [b] time x treatment interaction $p = 0.056$.

Table 3. Individual Exercise Performance Responses for Bout 2.

Subject:	CHO	BCAA	EAA
	Time to Fatigue (min)	Time to Fatigue (min)	Time to Fatigue (min)
1	28.25	**33.62**	27.6
2	**32.12**	25.02	22
3	**37.7**	28.85	30.67
4	**33.16**	16.83	23.5
5	20.37	**34.37**	15.67
6	15.63	**23.65**	11.2
7	21.33	**23.93**	20.77
8	19.1	**30.7**	26.6

Figure 1. Exercise Performance During Bout 2.

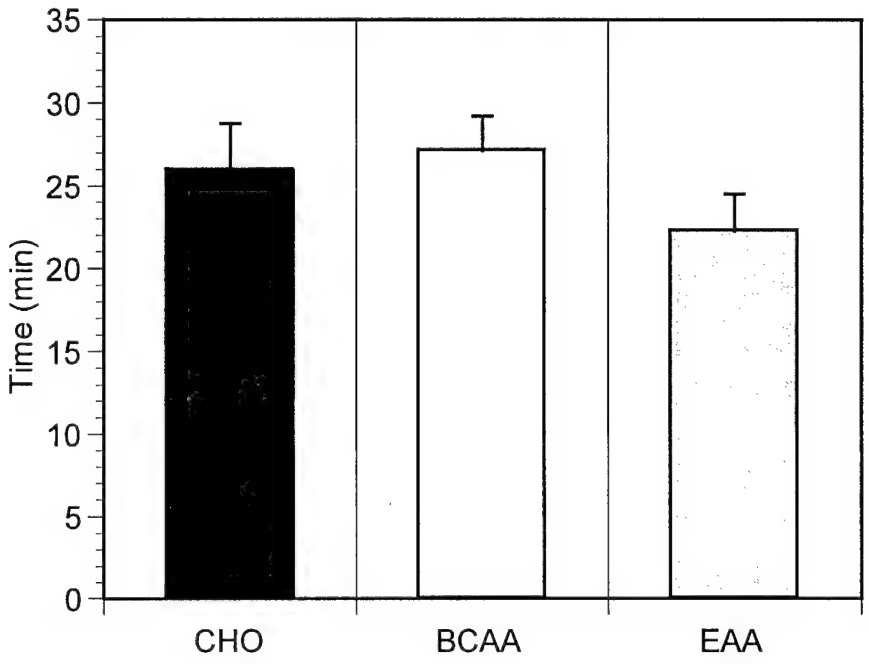
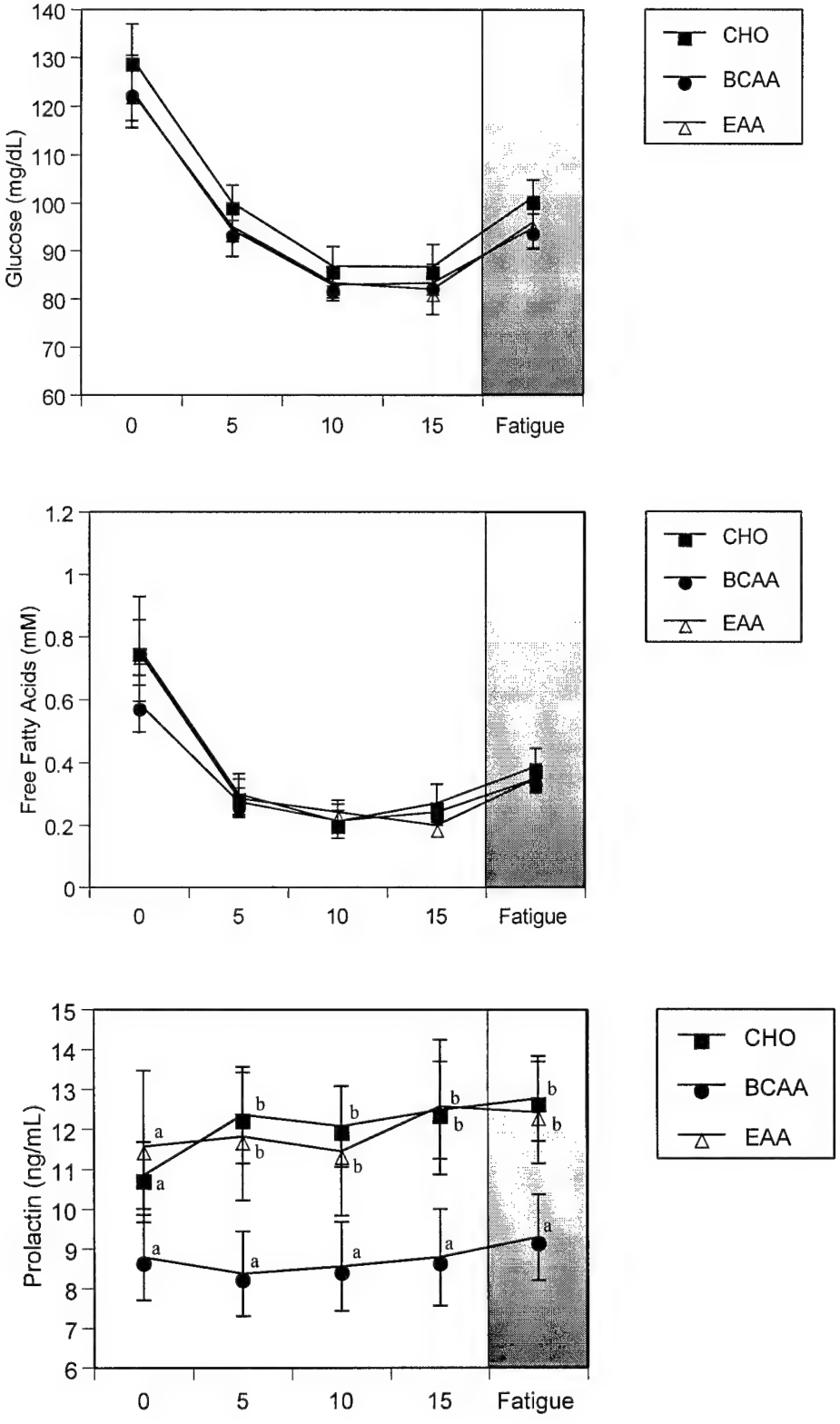


Figure 2. Serum Glucose, FFA and Prolactin Responses during Bout 2.



APPENDIX

TASK V: NUTRIENT DATABASE INTEGRATION LABORATORY

Words: 2858

Tables & Figures: 7

Photos: 0

Ref: 15

Contact: Champagne

Guarantor: Champagne

INCORPORATING NEW RECIPES INTO THE ARMED FORCES RECIPE FILE: DETERMINATION OF ACCEPTABILITY

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Key words: recipe development; Army recipes; low fat; recipe testing & development

ABSTRACT

As part of a project to lower fat, cholesterol and sodium in soldiers' diets, new ethnic and breakfast items were developed and standardized for 100 portions. Acceptability data were collected after initial recipe development, during recipe validation at a collaborating university, and in actual Army garrison. Acceptability was determined using a 9-point hedonic scale; products rating ≥6.0 in initial tests were prepared in garrison. Acceptability data were compared among test settings, ethnic categories, and food type. When grouped by ethnic categories, acceptability ratings varied more than when grouped by food type. Ratings varied most between development and validation settings (7.2 vs 6.6, $P<0.05$), and least between validation and actual Army settings (6.6 vs 6.6, ns). Since acceptability ratings were similar between validation and Army garrison, future recipe development may continue without additional testing at actual Army garrisons with considerable cost savings and more timely Armed Forces Recipe File additions.

INTRODUCTION

Since 1985, nutrition initiatives have been introduced into the Armed Forces Recipe Service, the Army Master Menu and the Army Food Service Program to provide soldiers with diets lower in fat, cholesterol and sodium. The Military Nutrition Division of the United States Army Research Institute of Environmental Medicine (USARIEM) conducted several garrison dining facility studies to assess soldiers' nutrient intakes (1,2,3). It was apparent from these studies that in order to achieve Army Nutrition Initiative goals of reducing fat to 30% of energy intake and cholesterol to 300 mg/day, extensive revision of Armed Forces Recipes would be required.

The demographic profile of the Army has changed in recent years. As of 1995, 13% of total Army personnel were female. The racial make-up included 62% white, 27% black, 5% Hispanic with the remaining 6% divided among Asian, native Indian, Alaskan Indian and other (4). Soldiers have increased their demand for the availability of ethnic food choices in dining facilities. This is most likely a reflection of the various backgrounds of individuals entering the military and gender differences in food preferences, as well as current eating trends in the United States. The Army is therefore trying to incorporate additional ethnic-based recipes into the Armed Forces Recipe File.

In 1990, the Military Nutrition Division, USARIEM, began a collaborative effort with Pennington Biomedical Research Center (PBRC) at Louisiana State University to modify Army garrison menus. The purpose of the project was to create healthful, nutritious menu items which moderate soldiers' fat, cholesterol, and sodium intakes. New ethnic-based recipes were developed to contain decreased fat, cholesterol and sodium levels. The goals for the project were

to develop low-fat ethnic and breakfast recipes, standardize them for 100 portions and test the recipes for acceptability in an actual Army garrison.

It has been standard practice for the recipe developers at Natick Research, Development, and Engineering (RD&E) to collect food preference and acceptance ratings before new recipes are added to the Armed Forces Recipe file. Natick RD&E uses two types of test panels - "technical" panels for quality, flavor and texture and "consumer" panels who participate in acceptance and attitude testing. Consumer panelists are asked to rate their acceptance of foods using a 9 point hedonic scale, where 9 equals "like extremely" and 1 equals "dislike extremely". Generally a new food item must receive a mean score of at least 6.0 to be considered acceptable. Because mean scores can be influenced by scores at either end of the spectrum, another criteria which may be used is the percentage of individuals who rate the product with a 6.0 or higher. Jezior et al. (5) reported that on a 9-point hedonic scale a mean of 6.0 corresponds to an acceptor size of 90% of the population. Ultimately, those products that are found acceptable under laboratory conditions are tested in actual military dining facility settings to determine whether the new food products are consumed in sufficient amounts to enter the system.

Judgments of the sensory and hedonic properties of food and food preferences are influenced by a variety of factors. Acceptability and consumption of food items depends on a complex interaction between the sensory properties of the food, the consumer expectations for it, its cognitive associations, convenience, and price (6). Gender and ethnic origin also influence food preferences. In a study of food preferences in military personnel, women had higher preferences for baked potatoes, green salads and fresh fruit while men had a higher preference for grilled meat (7). Researchers have documented a preference of cultures toward their own culture-specific foods (8) and examined cross-cultural flavor preferences (9). Recently a group

of investigators found that just labeling a food with an ethnic title increased perceived ethnicity and acceptability of the item (10).

Strong relationships between food choice and attitudes have been documented, especially toward foods with a high fat content (11, 12). Nutritional information has been found to increase consumers' hedonic response to some products (13). Solheim (14) studied the effects of information on fat content and sensory differences on like or dislike ratings by consumers of sausage. When sensory quality was similar, false information that the fat content of the 20%-fat sausage was 12% increased the hedonic rating while correct information on fat content decreased the rating.

There are many factors that influence acceptability of a food product and the ideal situation would be to test the acceptability of a food item in the population for which it is intended, but this is not always feasible. It is possible that groups of similar age, gender and ethnic origin would evaluate food items with similar acceptability ratings. The purpose of this study was to determine if acceptability data from a young college age population was similar to acceptability scores obtained from young soldiers in an actual Army garrison setting.

METHODS

The data were collected in three phases: recipe development, acceptability testing in a young population similar to Army personnel, and acceptability testing in an Army garrison population. The first phase consisted of recipe development at PBRC by a culinary research associate and nutrient analysis using Moore's Extended Nutrient Database (MENu) (Pennington Biomedical Research Foundation, 1997).

The first phase of this project was the development of recipes, which was conducted at

Pennington Biomedical Research Center (PBRC). Forty-seven new ethnic-based recipes were developed and divided into eight categories: American, breakfast, Cajun, Caribbean, Chinese, Indonesian, Italian and Mexican (Table 1). Each recipe was designated for a specific food type (bread, beef, dessert, fish, pasta, poultry, salad, starch, vegetable), as well as ethnic category. Initial testing for palatability and acceptability was conducted with a consumer panel. In addition to ethnic consideration, other criteria used for recipe development included nutritional adequacy to assure that the product was an appropriate fit for the nutrition initiatives, priced to fit within the meal cost constraints, and available for acquisition by the majority of military food service operations.

During phase two, each of the new recipes developed at PBRC were prepared as directed to yield 100 portions in the foods laboratory at Louisiana Tech University. Each of the recipes was evaluated for ease of preparation and clarity of method as outlined on the recipe cards during preparation. On 19 selected days, three new recipes per day were prepared and served in a cafeteria-like setting to individuals recruited from the campus. Subjects were able to select the food item they wished to eat from the recipes being tested for the project in a cafeteria type setting. Only foods developed for this project were included in the evaluation. For each food item selected, the subjects were asked to complete a food evaluation questionnaire.

Phase three was conducted in a military dining facility at Fort Polk, Louisiana. Over a three-week period, the new recipes were incorporated into the regular Army menu. PBRC culinary research associates were on site in the dining facility to train Army personnel in the correct techniques for preparing the new recipes. All 47 recipes were prepared and served at breakfast, lunch and dinner. Two food items from each food type which were already being served were selected to serve as controls. For control recipes, the intent was to collect average

ratings for each category so as to compare ratings for the new recipes. During the days selected for the study, evaluation questionnaires were distributed at each meal with a new menu item or a control item in the selected dining facility. These foods and their ratings are shown in Table 2. Every individual who selected a new menu item or control item was asked to complete an evaluation of the product. No information was provided regarding fat and sodium content of the recipes being evaluated.

Food Evaluation Questionnaire

The questionnaire contained closed-ended items related to demographic variables, typical use of product, and addition of condiments. In each of the three phases the recipes were evaluated with a single score for overall acceptance. The US military has a 40-year history of measuring like or dislike of food items to predict consumption. Hedonic evaluation of food started in 1950 with Peryam who developed the nine-point hedonic scale (15). The hedonic scale consists of nine separate phrases describing degrees of like and dislike. The scale ranges from 1 corresponding to "Dislike Extremely, to 5 "Neither Like or Dislike, and 9 "Like Extremely".

Subjects

Subjects for the first phase were faculty, staff, and students at the PBRC who volunteered to participate in routine taste tests of the recipes. They had no training in sensory analysis, but participated routinely in consumer acceptance tests of a wide range of food products. Age of the participants ranged from 18 years to about 50 years old, with 70% being younger than 29 years of age. Approximately 50% of those testing the recipes were male, 76% were white and 12% were black.

Subjects for the second phase were a convenience sample of faculty, staff, and students at Louisiana Tech University in Ruston, LA. Individual subjects changed on a daily basis, but the composition remained fairly constant. The majority (56%) was 29 years old or less, and 44% were male. Ethnic origin as completed by the participants was 82% white and 7% black, with the remainder indicating other ethnic backgrounds.

Testing for the third phase which occurred at a Fort Polk, LA dining facility included Army personnel who regularly ate their meals in that dining facility. They were asked to participate only after they had selected a modified food item or a control item from the serving line. Therefore individuals changed from day to day, but were similar in composition to the subjects in phase two. The majority of the Army personnel (76%) were 29 years old or younger and 90% were male. Most (62%) indicated their ethnic origin as white; 18% were black; and the remaining 20% were of other ethnic origins.

Data Analysis

All analyses were performed using SAS statistical package (1995). Descriptive statistics were calculated on demographic data. Mean hedonic responses for each new recipe were analyzed separately for each of the three phases of the study by ethnic and food type. Differences in acceptability scores of new recipes between Louisiana Tech and Fort Polk were assessed using t-tests. The percentage of subjects who rated a food product on the upper end of the hedonic scale ($\geq$6.0) was determined and chi square analyses were used to compare the proportion of mean ratings six and above by ethnic and food categories among the three test sites.

In order to investigate differences in food acceptability ratings with respect to test site, a

logistic regression model was employed. The binary response variable consisted of the proportion of mean acceptability ratings falling into the categories of "up to six" and "six and above." The analysis was conducted on proportions because they provided more robust comparisons. Test site was used as an explanatory variable. Results were obtained by ANOVA using a general linear model procedure. In addition, contrasts were written to examine specific comparisons between test sites. Grouping the recipes into two categories, ethnic foods and food types eased interpretations of the analysis.

RESULTS

Acceptability data were compared among the test sites by overall acceptability, ethnic categories, and food type. Acceptability of recipe items was higher at the development site (PBRC) than at the validation or actual Army site (Table 3). When grouped by ethnic category, the acceptability ratings were more variable than when grouped by food type. We found ratings varied most between the development and validation sites (7.2 vs 6.6, $P<0.05$), and least between the validation and actual Army site (6.6 vs 6.6, NS). Because ratings were similar between the validation and actual Army site, a more in-depth analysis was used to compare the two sites.

Although mean ratings varied least between the validation and actual Army setting, when t-tests were done between the two sites for 18-29 year olds, there were significant differences in ratings of breakfast foods. Four of the eight items included in the breakfast category had significantly higher ratings in the actual Army setting. There were also significant differences among dessert, salad, and starch categories. For the majority of food items, the mean scores were higher in the Army garrison setting than at Louisiana Tech (Table 3).

The percentages of acceptable ($\geq$6.0) and unacceptable (<6.0) scores for each testing site

are presented in Table 4A. It is evident that the percentage of acceptable dishes was more similar between Louisiana Tech and Fort Polk than at Pennington, whose acceptability percentage was higher. Table 4B contains mean hedonic ratings for the ethnic and food type categories for 18-29 year olds at Louisiana Tech and Fort Polk. For ethnic comparisons, the only difference noted was for breakfast dishes for which the acceptability rating was higher at Louisiana Tech. A few differences were noted in food type analysis. Dessert and starch dishes were rated more acceptable at Louisiana Tech, while salad dishes were found more acceptable at Fort Polk.

Chi square analyses revealed variations in ethnic foods and food types were generally due to differences at PBRC compared with either Louisiana Tech or Fort Polk (Tables 5A and 5B). These variations were observed for all ethnic dishes except for Caribbean and Italian dishes. With respect to comparisons between Louisiana Tech and Fort Polk, significant differences were noted only in food type, specifically in pasta, poultry, salad, starches, and vegetables. Variations were noted based on the proportion of mean ratings falling into the two response acceptability categories, "up to six" and "six and above." For those contrasts that are significant, a "+" or "-" indicates the direction of the difference. For example, if the contrast of Louisiana Tech vs. Fort Polk is significant, then a "+" indicates that the proportion of ratings were significantly higher for Louisiana Tech than for Fort Polk. A "-" indicates that the proportion of ratings were significantly higher for Fort Polk than for Louisiana Tech.

DISCUSSION

The development site (PBRC) had acceptability scores differing greatly from the actual Army test site, quite possibly due to a difference in gender, age, and ethnicity of participants evaluating the food items. The validation site (Louisiana Tech) was more closely matched to the

Army site in terms of subject demographics than PBRC, thus providing scores that were similar to the Army scores.

When comparing acceptability scores of individual items between the validation and Army sites, 19 food items had scores that were different. However, only five items from those that were different at the Army site were below the 6.0 acceptability level, indicating that acceptance remained high in most items evaluated. Percentages of acceptable and unacceptable recipe items varied by site in some categories. Comparisons of food by ethnic categories revealed that the Army site was more critical of Caribbean, Mexican, and breakfast foods; comparisons of food type showed that the Army was also more critical of beef, bread, dessert, and poultry recipes.

There are several possible reasons for differences in acceptability scores of specific recipes at the validation and Army site. They could have resulted from: 1) differences in pre-preparation, holding, and serving of food; 2) types of other foods offered in combination with the test items; or 3) gender and ethnicity differences between participants at both sites. Another consideration may be food selection rates between items, given greater choice availability. Since acceptability ratings were so similar between validation and Army garrison, we anticipate that future recipe development can continue without additional testing at an actual Army garrison allowing for considerable cost savings and more timely additions to the Armed Forces Recipe File.

As a future consideration, periodic evaluations that more closely match the validation site with an Army site in demographic characteristics, such as age, gender, and ethnicity of individuals consuming the foods, would confirm the acceptability of new recipes as the population mix of the military changes. Evaluation of newly developed recipes may also need to

be conducted at alternate sites, such as Navy ships, to determine feasibility of their inclusion in the Armed Forces Recipe File for use by the other services that rely on the recipe file for meal preparation for military personnel.

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Table 1. Ethnic Groupings of Developed Recipes

AMERICAN
- Apple Betty
- Garlic Cheese Potatoes
- German Potatoes
- Horseradish Potatoes
- Roasted Vegetable Salad
- Tomato Salad
- Turkey Chili
- Turkey Stroganoff
- Turnips and Greens

BREAKFAST
- Biscuits
- Breakfast Burrito
- Breakfast Potatoes
- Eggs Florentine
- Jalapeno Cheese Grits
- Oatmeal Raisin Bar
- Three Berry Muffin
- Turkey Sausage

CAJUN
- Bread Pudding
- Cajun Meatloaf
- Chicken Jambalaya
- Chicken Sauce Piquant
- Eggplant Tomato Salad
- Fish Piquant
- Red Beans with Turkey Sausage
- Summer Squash

CARIBBEAN
- Caribbean Jerk Chicken
- Caribbean Pot Roast
- Jamaican Rum Chicken
- Okra Melange

CHINESE
- Beef with Broccoli
- Chicken with Orange Glaze
- Cucumber Salad
- Oriental Chicken Salad
- Rolled Fish
- Vegetable Rice

INDONESIAN
- Fish and Mushrooms
- Thai Beef Salad

ITALIAN
- Italian Potatoes
- Pasta Primavera
- Pasta Provencal
- Pasta Putanesca

MEXICAN
- Chicken Fajitas
- Mexican Black Beans
- Mexican Cornbread
- Seven Bean Salad
- Southwestern Rice
- Vegetarian Burrito

Table 2. Acceptability ratings of control recipes (mean ± standard deviation)

Armed Forces Recipe File Recipe Identification Number	Recipe Name	Acceptability Score (mean ± standard deviation)
G-29	Pineapple Cake	7.5 ± 1.8
Q-41	Peas	6.8 ± 1.4
M-34	Macaroni Salad	6.6 ± 1.8
L-142	Chicken	6.7 ± 1.4
L-89	Pork Chops	7.4 ± 1.2
L-35-2	Meatloaf with Tomatoes	7.0 ± 1.4
E-8	Rice	6.6 ± 1.8
Q-50	Oven Browned Potatoes	6.0 ± 2.1
F-10	Scrambled Eggs	6.5 ± 1.6
Q-46	Breakfast Potatoes	6.5 ± 1.7

Table 3. Overall averages by centers for ethnic and food types

Center	Ethnic	Food Type
Pennington	7.26 [a]	7.24 [a]
Louisiana Tech	6.61 [b]	6.63 [b]
Fort Polk	6.62 [b]	6.68 [b]

[ab] Means within columns with different superscript letters are different ($P<0.05$)

Table 4A. Comparison of acceptability ratings

Center	Unacceptable <6.0	Acceptable 6.0+
Pennington (PBRC)	21.3%	78.7%
Louisiana Tech	34.9%	65.1%
Fort Polk	33.2%	66.8%

Table 4B. Comparison of hedonic ratings (means ± standard errors) for 18-29 yr olds at the test sites.

	Louisiana Tech		Fort Polk		T-test
	N	Mean ± S.E.	N	Mean ± S.E.	P value
Ethnic					
American	454	6.26 ± .10	162	6.14 ± .19	0.977
Breakfast	421	6.73 ± .08	164	6.18 ± .16	0.006
Cajun	433	7.08 ± .08	195	6.88 ± .13	0.260
Caribbean	187	7.21 ± .11	53	6.92 ± .22	0.336
Chinese	357	6.76 ± .10	167	6.57 ± .15	0.473
Indonesian	111	6.80 ± .13	0	-- ± --	--
Italian	217	7.07 ± .09	65	6.78 ± .22	0.515
Mexican	323	6.86 ± .10	158	6.72 ± .15	0.523
Food Type					
Bread	167	6.72 ± .14	95	6.33 ± .20	0.067
Beef	226	7.31 ± .09	77	7.04 ± .20	0.688
Dessert	115	7.28 ± .15	57	6.56 ± .27	0.025
Fish	174	6.70 ± .14	64	6.48 ± .23	0.389
Pasta	152	7.00 ± .11	44	7.02 ± .26	0.502
Poultry	558	7.19 ± .06	265	6.87 ± .11	0.087
Salad	446	6.11 ± .10	84	6.62 ± .23	0.015
Starch	524	7.05 ± .07	264	6.45 ± .13	0.002
Vegetable	132	6.02 ± .17	34	6.62 ± .33	0.082

Table 5A. Chi square analysis results for ethnic comparisons between centers.

	Chi-Square	P-value	Direction
American:			
Overall	52.37	0.0000	
Pennington vs Ft. Polk	33.41	0.0000	+
Pennington vs La. Tech	49.13	0.0000	+
La. Tech vs Ft. Polk	00.06	0.8058	
Breakfast:			
Overall	18.61	0.0001	
Pennington vs Ft. Polk	17.68	0.0000	+
Pennington vs La. Tech	12.05	0.0005	+
La. Tech vs Ft. Polk	02.20	0.1383	
Cajun:			
Overall	9.44	0.0089	
Pennington vs Ft. Polk	3.05	0.0806	
Pennington vs La. Tech	9.40	0.0022	+
La. Tech vs Ft. Polk	1.42	0.2335	
Caribbean:			
Overall	4.23	0.1206	
Pennington vs Ft. Polk	3.07	0.0799	
Pennington vs La. Tech	3.74	0.0531	
La. Tech vs Ft. Polk	0.00	0.9718	
Chinese:			
Overall	18.72	0.0001	
Pennington vs Ft. Polk	11.65	0.0006	+
Pennington vs La. Tech	18.17	0.0000	+
La. Tech vs Ft. Polk	00.23	0.6342	
Indonesian:			
Overall	20.59	0.0000	
Pennington vs Ft. Polk	14.14	0.0002	+
Pennington vs La. Tech	20.48	0.0000	+
La. Tech vs Ft. Polk	01.10	0.2951	
Italian:			
Overall	1.55	0.4614	
Pennington vs Ft. Polk	0.02	0.8917	
Pennington vs La. Tech	0.79	0.3744	
La. Tech vs Ft. Polk	1.25	0.2643	
Mexican			
Overall	10.80	0.0045	
Pennington vs Ft. Polk	08.59	0.0034	+
Pennington vs La. Tech	09.46	0.0021	+
La. Tech vs Ft. Polk	00.02	0.8957	

Table 5B. Chi square analysis results for food type comparisons between centers.

	Chi-Square	P-value	Direction
Beef:			
Overall	18.38	0.0001	
Pennington vs Ft. Polk	18.11	0.0000	+
Pennington vs La. Tech	13.05	0.0003	+
La. Tech vs Ft. Polk	01.32	0.2514	
Bread:			
Overall	1.82	0.4022	
Pennington vs Ft. Polk	1.46	0.2266	
Pennington vs La. Tech	0.14	0.7098	
La. Tech vs Ft. Polk	1.18	0.2767	
Dessert:			
Overall	8.16	0.0169	
Pennington vs Ft. Polk	8.13	0.0044	+
Pennington vs La. Tech	4.13	0.0422	+
La. Tech vs Ft. Polk	1.59	0.2072	
Fish:			
Overall	29.16	0.0000	
Pennington vs Ft. Polk	23.22	0.0000	+
Pennington vs La. Tech	28.17	0.0000	+
La. Tech vs Ft. Polk	00.10	0.7490	
Pasta:			
Overall	6.40	0.0408	
Pennington vs Ft. Polk	1.65	0.1994	
Pennington vs La. Tech	0.91	0.3392	
La. Tech vs Ft. Polk	6.39	0.0115	-
Poultry:			
Overall	23.32	0.0000	
Pennington vs Ft. Polk	21.83	0.0000	+
Pennington vs La. Tech	05.17	0.0230	+
La. Tech vs Ft. Polk	10.27	0.0013	+
Salad:			
Overall	68.15	0.0000	
Pennington vs Ft. Polk	06.42	0.0113	+
Pennington vs La. Tech	61.41	0.0000	+
La. Tech vs Ft. Polk	20.70	0.0000	-
Starches:			
Overall	8.36	0.0153	
Pennington vs Ft. Polk	7.90	0.0049	+
Pennington vs La. Tech	1.52	0.2174	
La. Tech vs Ft. Polk	4.04	0.0445	+
Vegetables:			
Overall	29.77	0.0000	
Pennington vs Ft. Polk	01.33	0.2495	
Pennington vs La. Tech	25.57	0.0000	+
La. Tech vs Ft. Polk	10.41	0.0013	-

APPENDIX

TASK VI: ENHANCING MILITARY DIETS

Time/Flow Analysis: Week 1

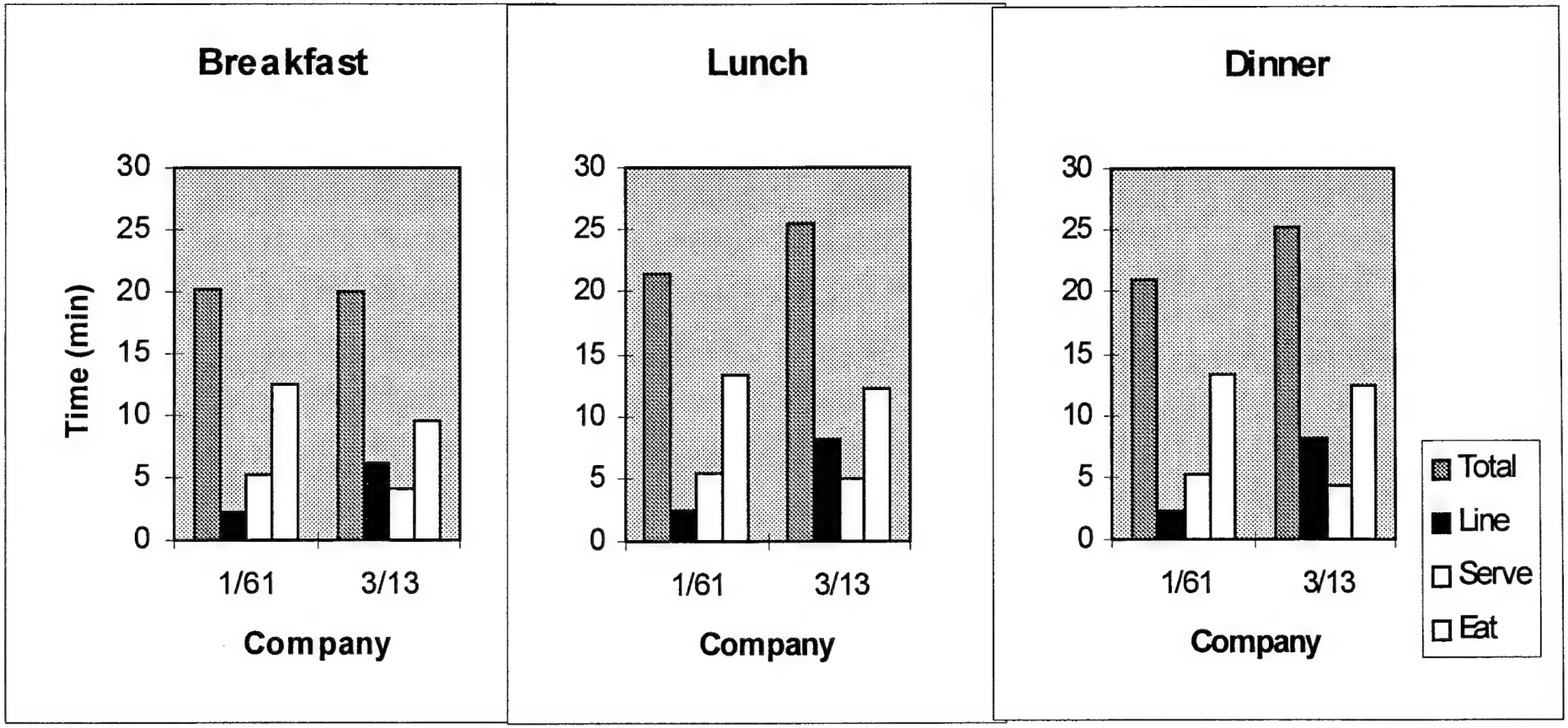


FIG 1

Time/Flow Analysis: Week 8

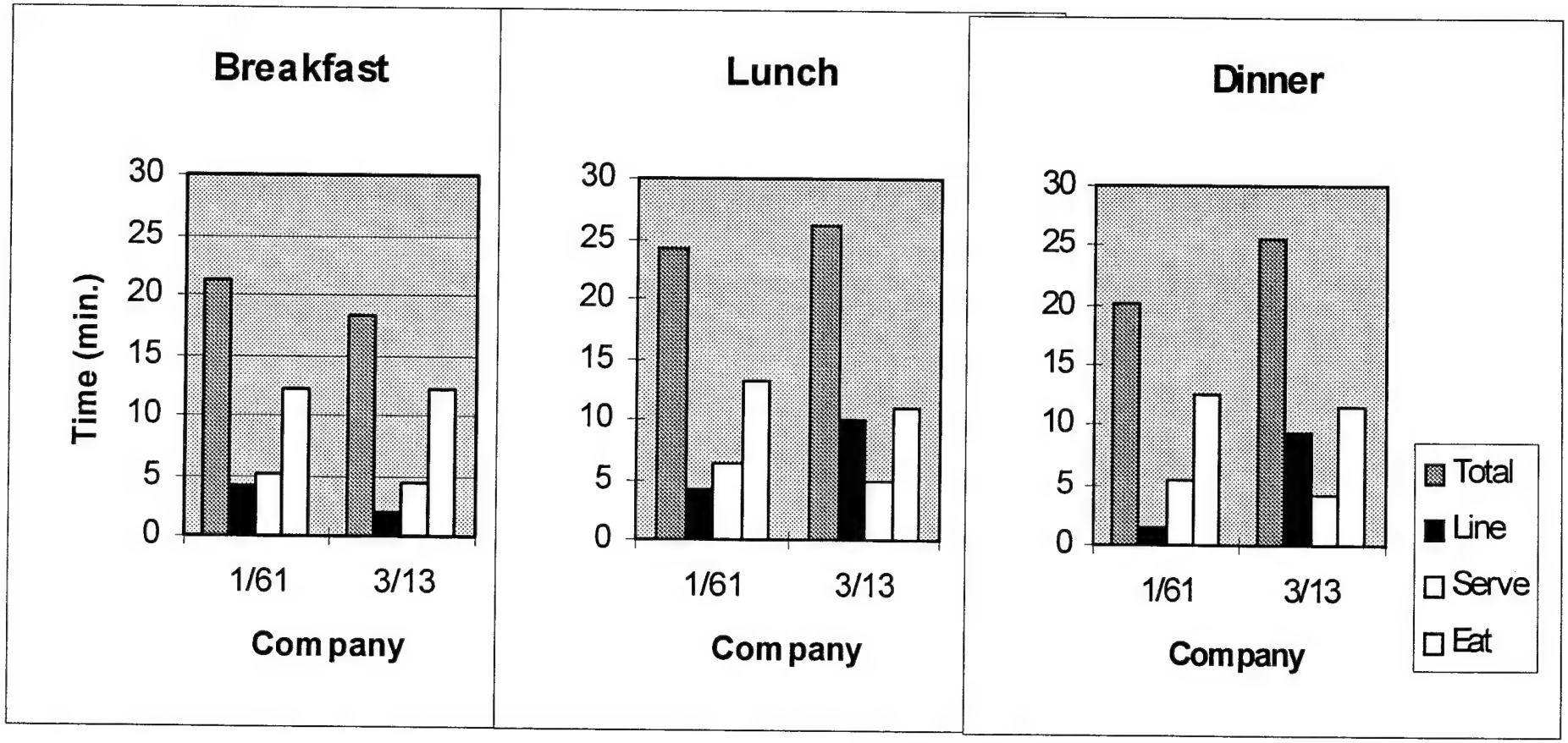


FIG 2

FIG 3

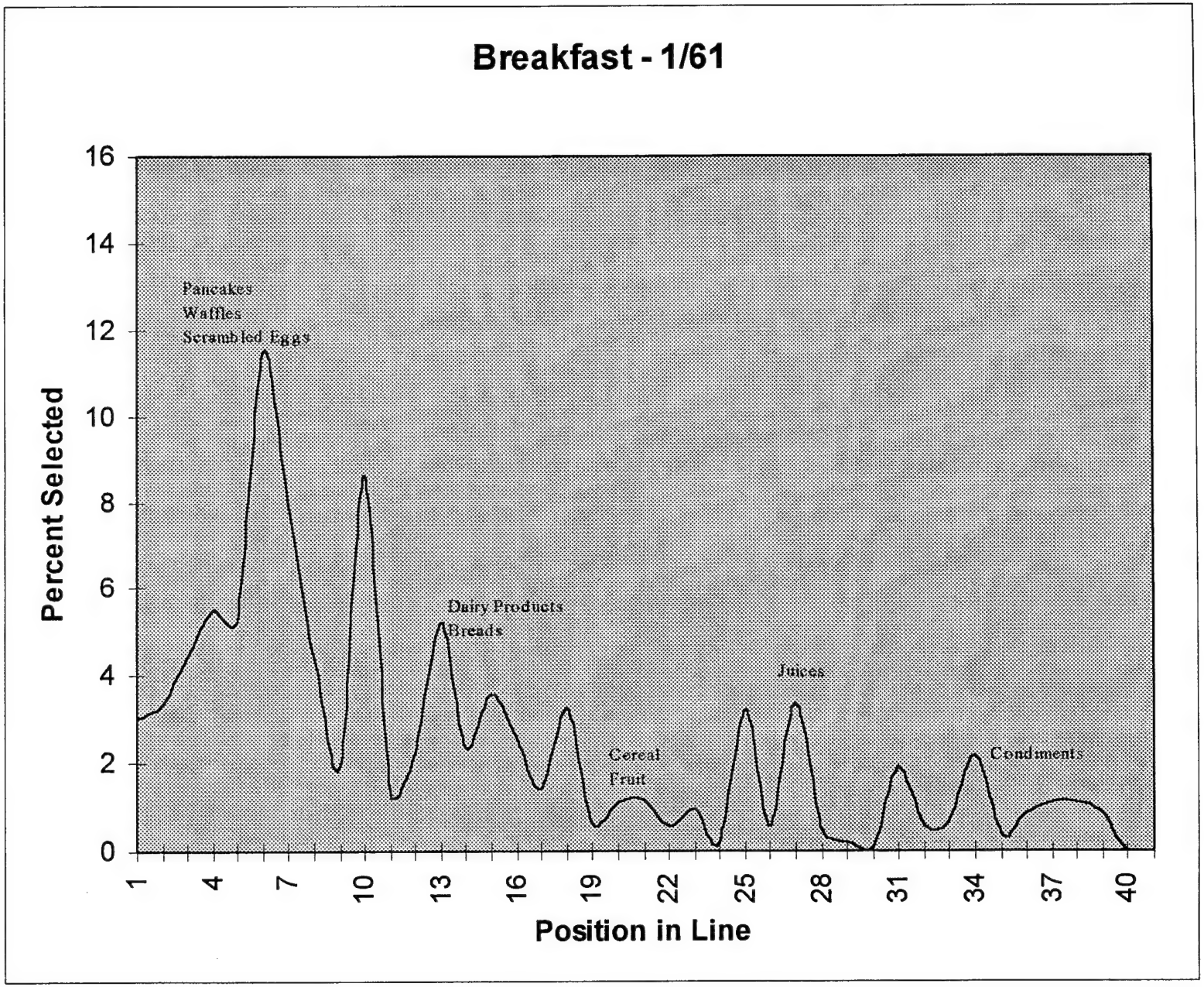

Breakfast - 1/61
Percent Selected
Position in Line
Pancakes
Waffles
Scrambled Eggs
Dairy Products
Breads
Cereal
Fruit
Juices
Condiments
0
2
4
6
8
10
12
14
16
1
4
7
10
13
16
19
22
25
28
31
34
37
40

FIG 4

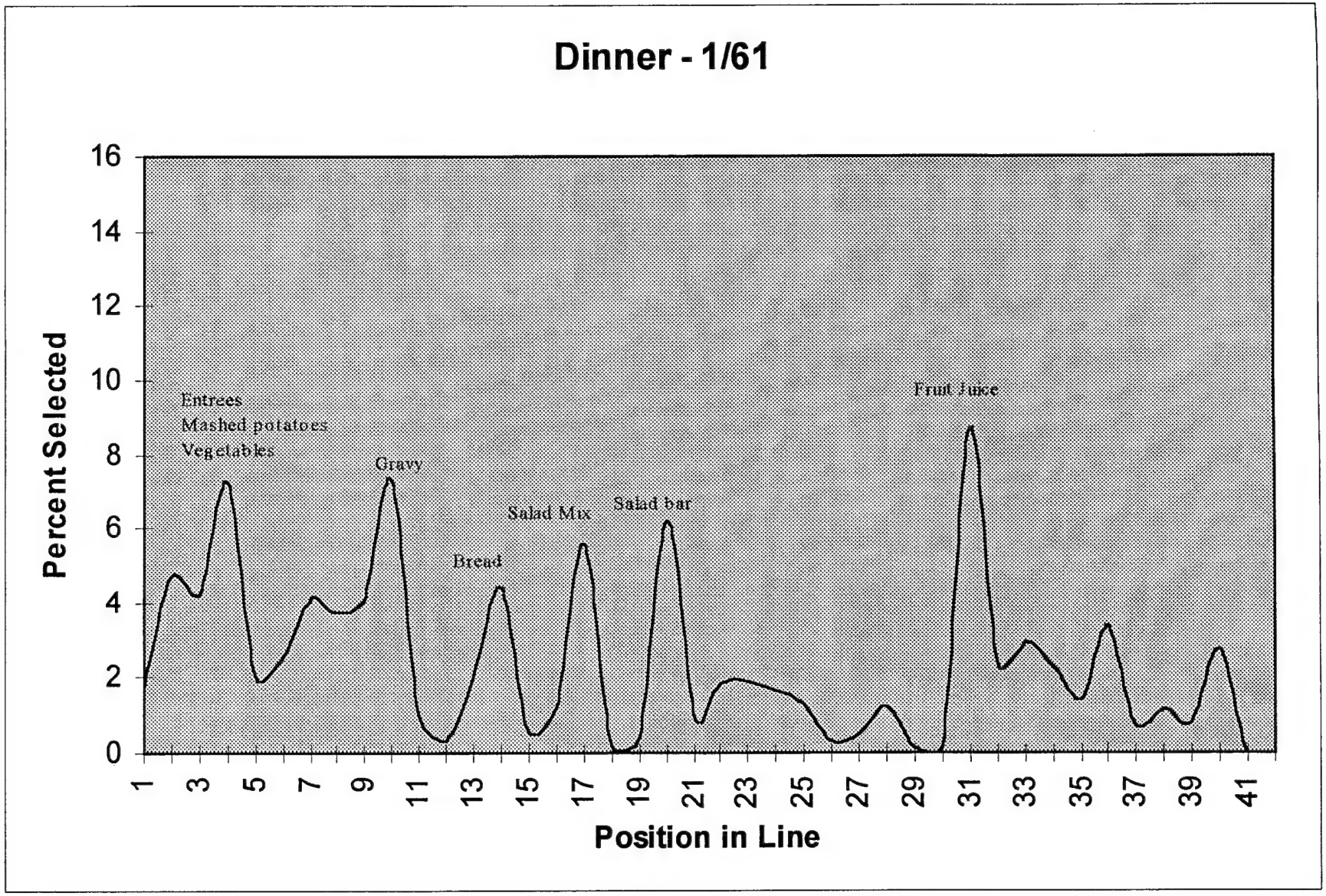
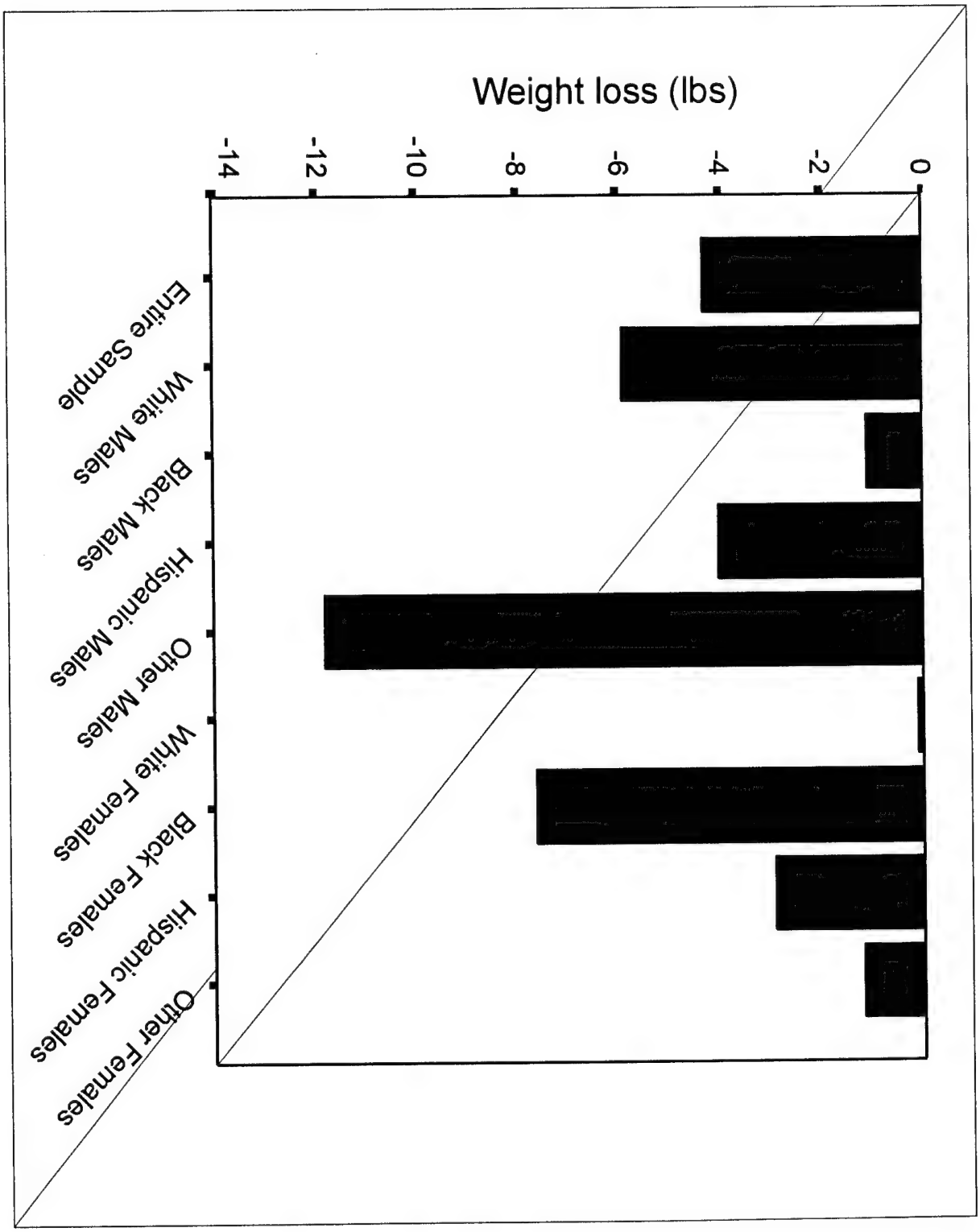

Dinner - 1/61
Percent Selected
Position in Line
Entrees
Mashed potatoes
Vegetables
Gravy
Bread
Salad Mix
Salad bar
Fruit Juice
0
2
4
6
8
10
12
14
16
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
33
35
37
39
41

FIG 5


Weight loss (lbs)
-14
-12
-10
-8
-6
-4
-2
0
Entire Sample
White Males
Black Males
Hispanic Males
Other Males
White Females
Black Females
Hispanic Females
Other Females

APPENDIX

TASK VII: STRESS, NUTRITION AND IMMUNE FUNCTION LABORATORY

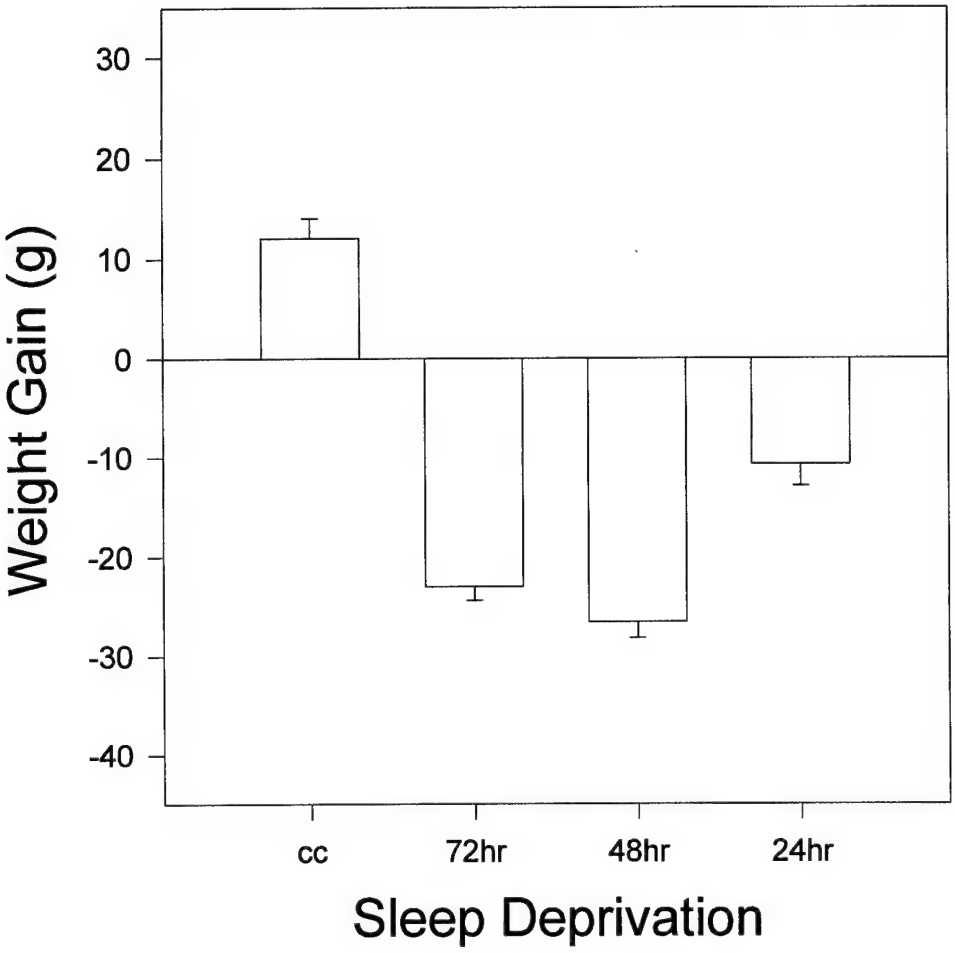


Figure 1. Rats subjected to sleep deprivation experience weight loss. Four rats per group were subjected to 24, 48 or 72 hours of sleep deprivation in a pedestal tank. An additional group of 4 rats were kept in their cages as controls (CC). Each rat was weighed before and after sleep deprivation and the difference between the two measurements is shown in the figure. The bars represent the average (and standard deviation) weight gain (loss).

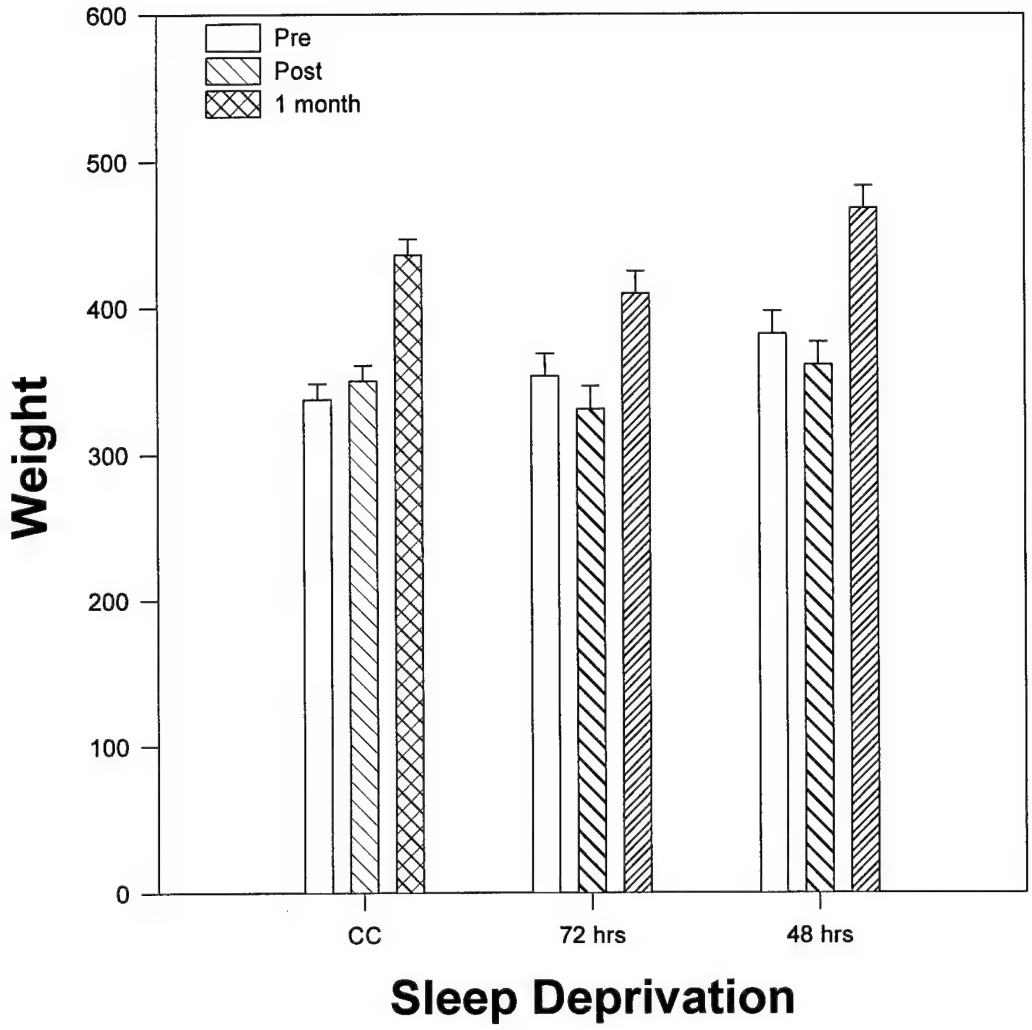


Figure 2. Rats subjected to 72 hours of sleep deprivation have prolonged weight loss. Rats were subjected to 48 or 72 hours of sleep deprivation using a pedestal tank. The rats were weighed prior to sleep deprivation (Pre), immediately after sleep deprivation (Post), and one month later. The results represent the average and standard deviation of 4 rats per group.

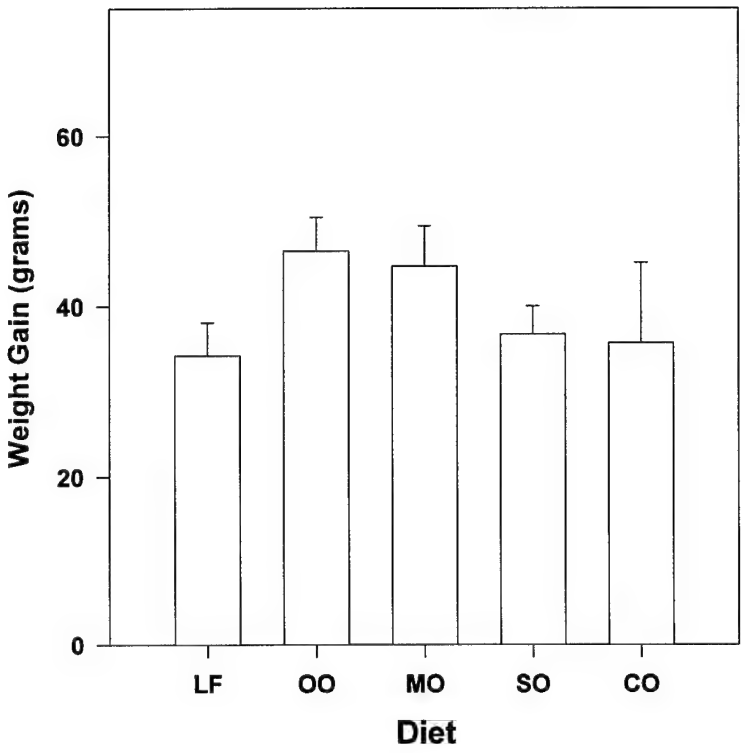


Figure 3. Effect of diets on weight gain after two weeks on diet. Groups of 8 rats each were fed either a low fat diet (LF), a safflower oil diet (SO) high in n-6 polyunsaturated fatty acids (PUFA), an olive oil diet (OO) high in monosaturated fatty acids, a coconut oil diet (CO) high in saturated fatty acids, and a menhaden oil (MO) diet high in n-3 PUFA for two weeks and weighed. The results represent the average and standard deviation for each diet group

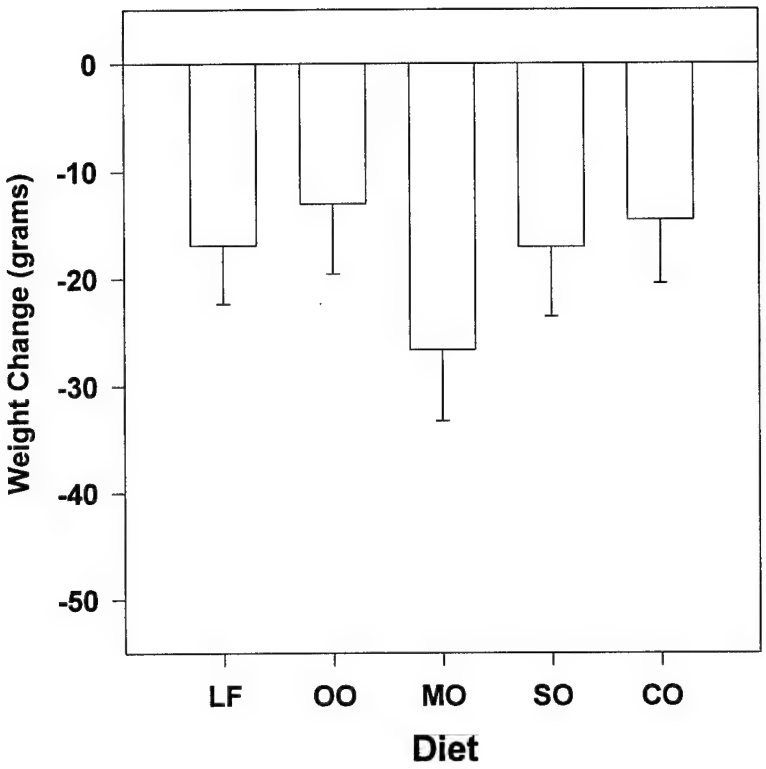


Figure 4. Sleep deprivation results in a similar weight loss amongst the different diet groups. The rats from Figure 3 were subjected to 72 hours of sleep deprivation and weighed again. Results represent the average weight change (loss) and standard deviation of 4 rats per group. Cage control rats did not loose any weight during the same period (data not shown).

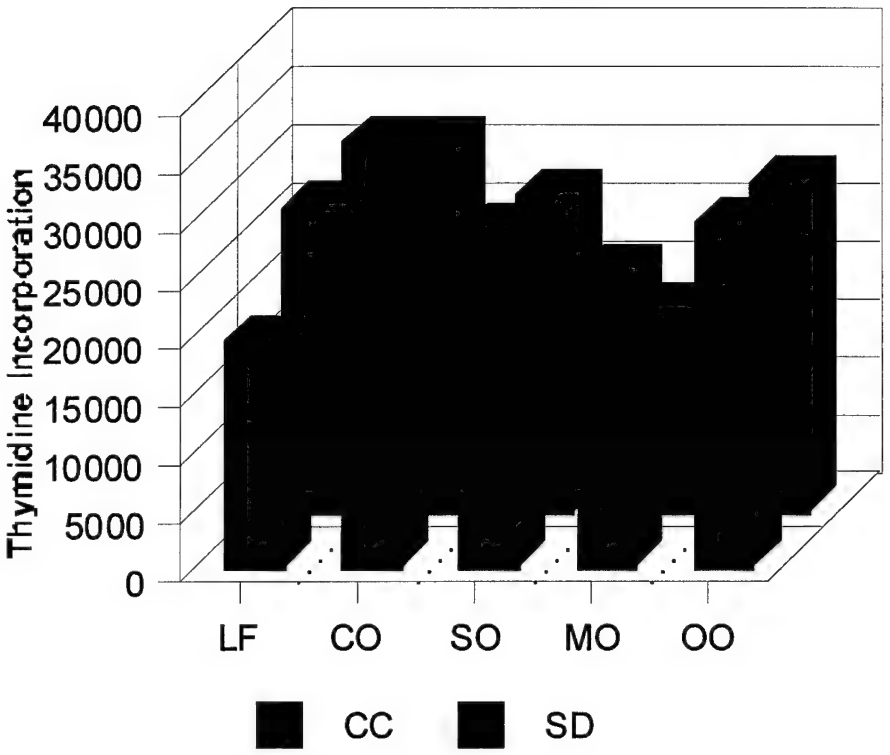


Figure 5. Dietary fat modulates sleep deprivation-induced suppression of the lymphoproliferative response to Con A. Rats (4/group) were pre-fed their respective diets for 2 weeks prior to sleep deprivation. Sleep deprived (SD) rats were kept in pedestal tanks for 48 hours. Cage control (CC) rats were kept in the same room. All rats had access to liquid diet of the same formulation during the period of sleep deprivation. The rats were sacrificed and necropsied immediately after the sleep deprivation period. Splenocyte cultures were incubated with concanavalin A for 72 hours and then proliferation measured using ^{3}H-thymidine incorporation.

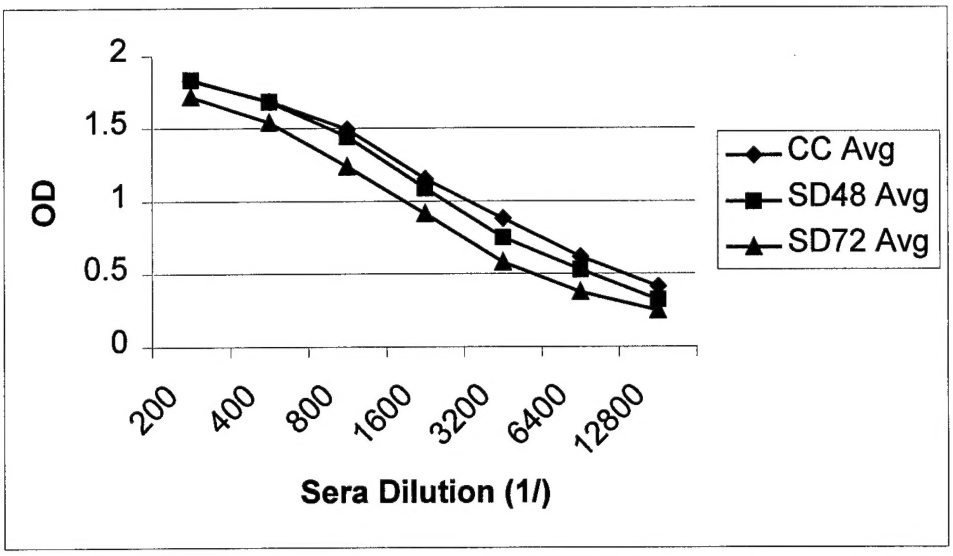


Figure 6. Sleep deprivation reduces the antibody response to a vaccine. Rats were vaccinated with 100 μg of KLH mixed with alum immediately prior to sleep deprivation for 48 or 72 hours. Cage control rats received the same vaccine but were not sleep deprived. Sera samples were collected one month post vaccination and antibody titers determined using an ELISA. The results represent the average of 4 rats per group.

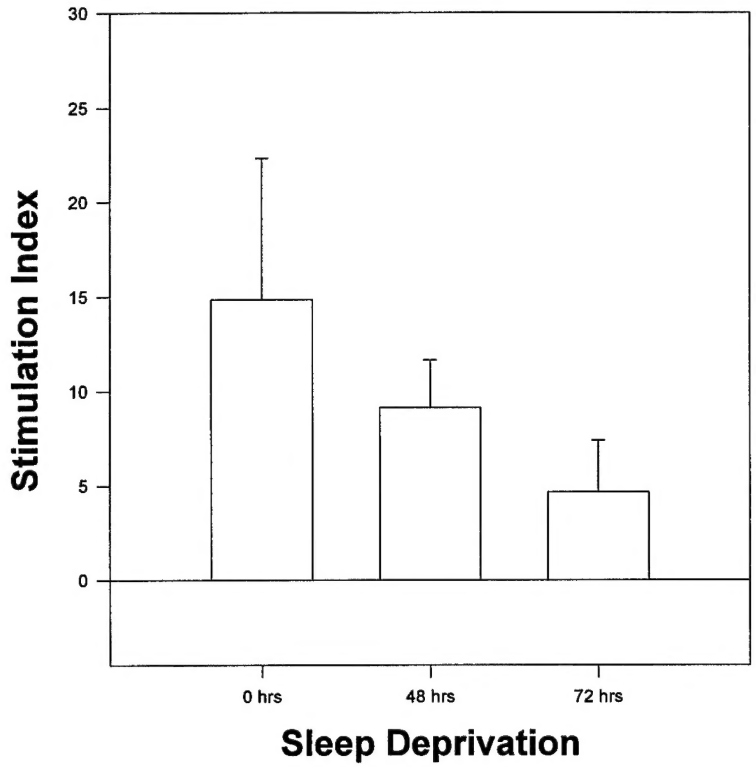


Figure 7. Sleep deprivation results in a diminshed lymphoproliferative response to a vaccine antigen. Rats were vaccinated with 100 μg of KLH mixed with alum immediately prior to sleep deprivation for 48 or 72 hours. Cage control rats received the same vaccine but were not sleep deprived. One month after sleep deprivation the rats were necropsied and their splenocytes incubated with KLH (3 μg/ml) for 120 hours. The proliferative response was measured using ^{3}H-thymidine incorporation and the results presented as stimulation indices. The results represent the average and standard deviation of four rats per group.

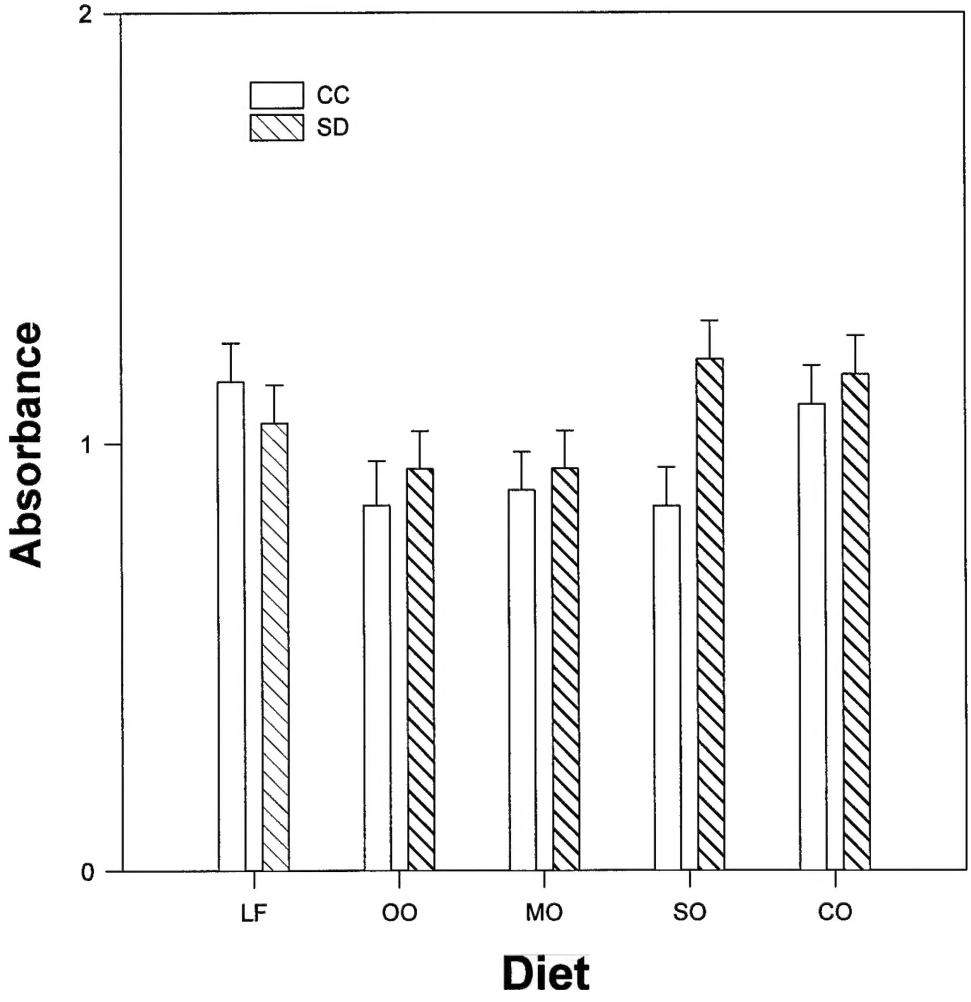


Figure 8. A high fat diet reduces the negative effect of sleep deprivation on antibody production following vaccination. Sera samples were collected one month after sleep deprivation and diluted 1/200 prior to ELISA measurement for KLH-specific antibodies. The results represent the mean and standard deviation for 4 rats per group.

APPENDIX

TASK VIII: METABOLIC UNIT PROJECT